MASTER OF SCIENCE

Investigating the effect of phosphodiesterase inhibitors on human sperm motility and function

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Chapter 1: Introduction

1.1 Development of Assisted Reproductive Technology (ART)

Since its inception there has been a remarkable increase in the development and practice of ART including both in-*vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) treatment (Figure 1.1). Correlated with an increase in the number of babies born following both IVF and ICSI treatment, ART has rapidly expanded in both its use and its acceptance (Tesarik et al., 1992). For example, the number of infants born from ART procedures (including IVF, ICSI, GIFT and ZIFT) has doubled from 30,629 in 1999 to 61,426 in 2008 (CDC, 2010). The revolutionary techniques of ART have allowed a solution for many couples. This progress includes an advance in the quality of treatment, such as the improvement of techniques used, from ICSI and surgical sperm retrieval to embryo cryopreservation. Due to such progress the success rate of ART has also increased. For example, the percentage of transfers resulting in live births increased from 11.5% in 1999 to 22% in 2008 for elective single embryo transfer (eSET) and from 30.9% in 1999 to 43.7% in 2008 for double embryo transfer (DET) (Figure 1.2). Although the success rate of ART has increased, the type of treatment provided depends on individual circumstances. Some of these circumstances include woman's age or sperm quality as the cause of infertility. With ICSI representing almost half (47%) of all ART treatment in the UK in 2006, (Kurinczuk, 2010) this indicates that the male element is in fact the most common infertility problem between couples undergoing ART. However, there is limited knowledge of the causes of male infertility.

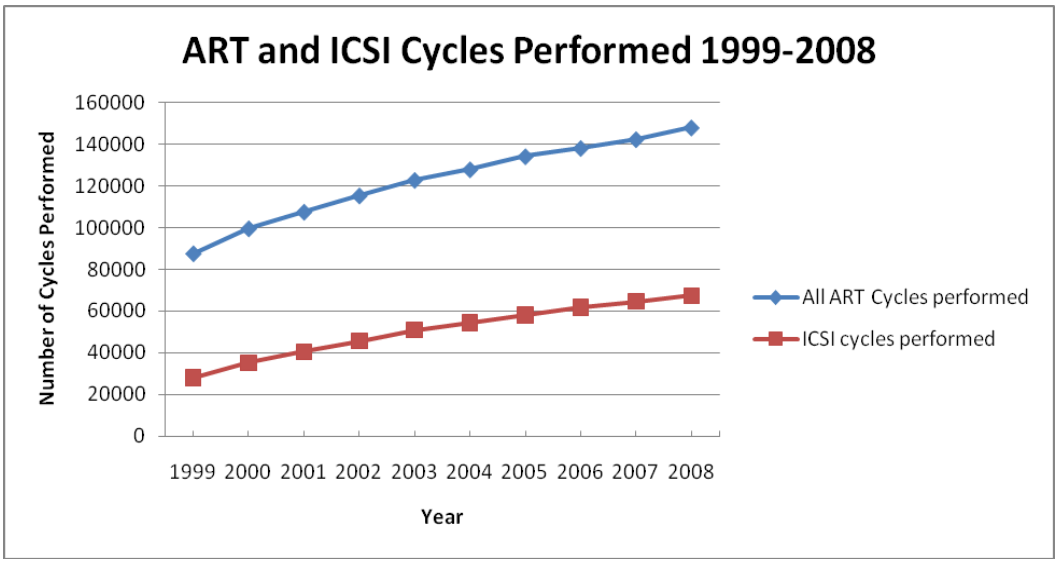

Figure 1.1 Interpretation of data from CDC (2010) ART Trends 1999-2008. Representing the number of all ART cycles (including IVF, ICSI, GIFT and ZIFT) performed compared with ICSI cycles alone using fresh non-donor oocytes in the US.

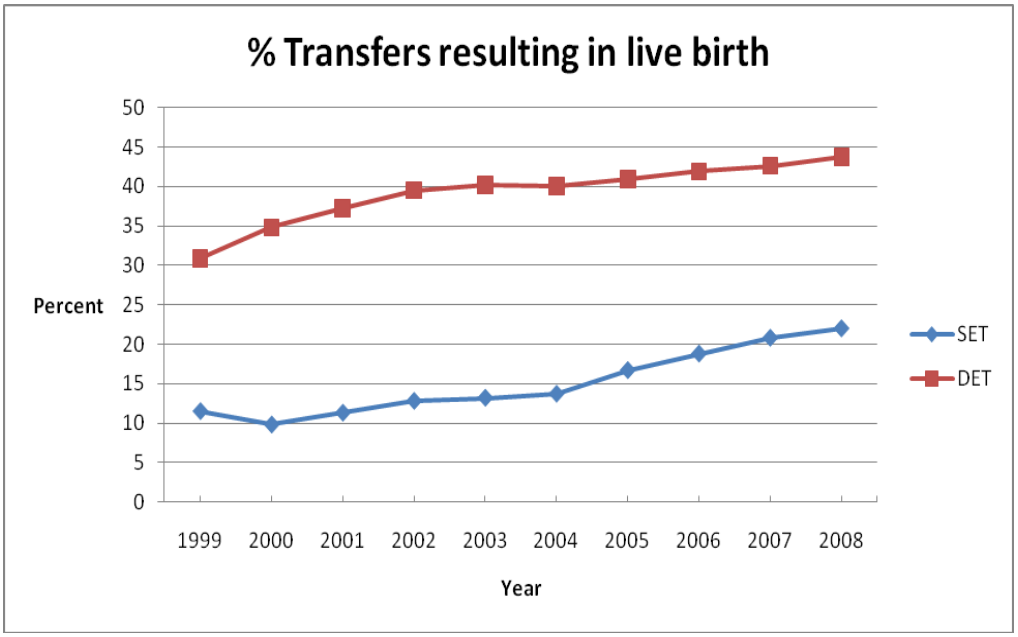

Figure 1.2 Interpretation of data from CDC (2010) ART Trends 1999-2008. Representing the percentage of elective SET and DET transfers resulting in live births using fresh non-donor oocytes in the US.

1.2 Causes of male infertility

Generally, infertility is viewed as a disease requiring medical treatment (Zegers-Hochschild et al., 2009). Infertility is recognised as the inability of a couple to conceive after two years of contraceptive free intercourse (NICE). This may be divided into primary and secondary infertility. The latter being couples who have already had children and are unable to conceive. Nevertheless, both face the reality of infertility. On examining infertility trends over recent years, there is a rise in the occurrence of male factor infertility presented by the increase in ICSI (Figure 1.1). The number of ICSI procedures performed more than doubled from 28,090 in 1999

to 67,328 in 2008 in the US (CDC 2010). The most recent HFEA data also suggests that male factor infertility is predominantly the main diagnosis in patients seeking treatment compared to other factors (Figure 1.3). With further analysis, it would appear that ICSI is used more than expected and not only for male factor infertility. In the US in 2008, ICSI was carried out on 67,258 fresh non-donor cycles. Out of these cycles only 47.1% were diagnosed with male factor infertility with the majority (52.9%) having no male factor problems. However, procedures such as IUI, IVF or ICSI are dependent upon the parameters used for the diagnosis of male factor infertility. These vary between clinics and in turn, so do procedures. Results from ACU Ninewells in Dundee demonstrate the reality of male factor infertility. Male factor is primarily the reason for infertility from all ICSI cycles in 2010 with 84.7% embryo transfers originating from male factor infertility and only 6.9% female factor (Figure 1.4). These figures demonstrate a high proportion of male factor infertility in the ICSI population.

The cause of male infertility is unknown in roughly 50% of cases (Oehninger and Franken, 2006). Various factors come under the heading of unknown and these elements of infertility are classed as unexplained. It is surprising that little is known as to what causes male infertility. The term ‘unexplained’ covers a range of sperm problems such as concentration, morphology, motility or sperm vitality. There are also abnormalities in sperm function, including the inability to bind to and penetrate the zona pellucida (Cahill and Wardle, 2002). For this the sperm must have progressive motility (Simon and Lewis, 2011) and the ability to acrosome react (Krausz et al., 1994) to enable successful sperm-oocyte interaction. Human sperm

motility defects are associated with 82% of cases with male infertility (Curi et al., 2003; Chemes, 2000), yet little is known about the cause.

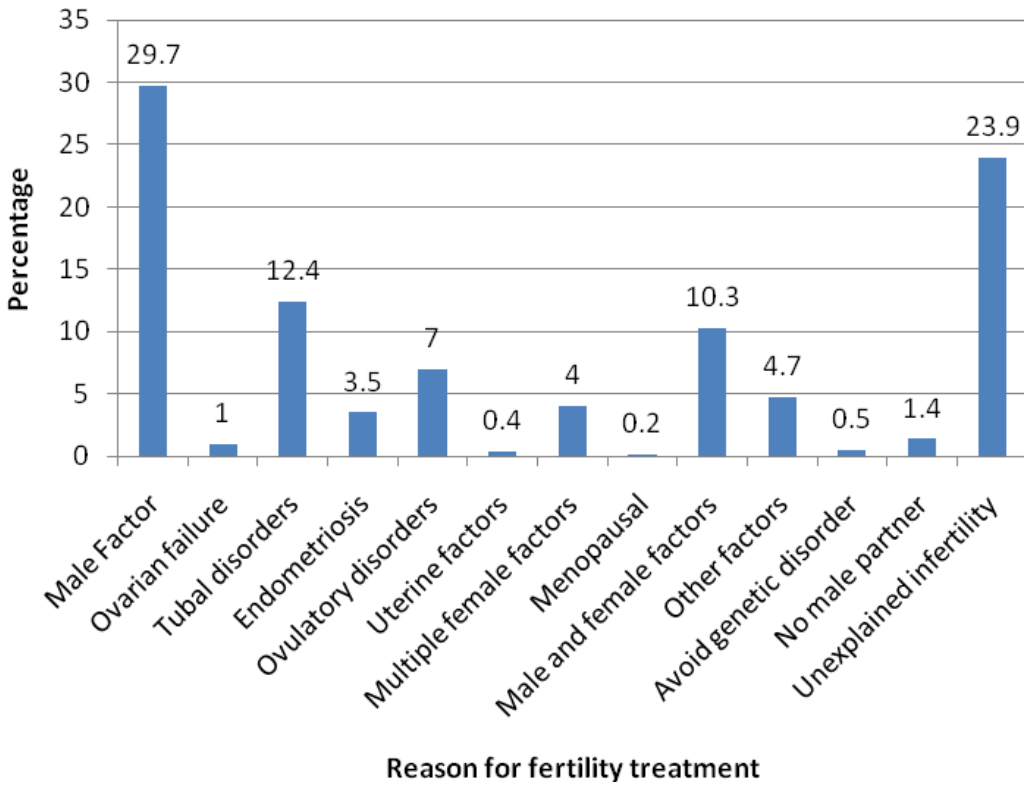

Figure 1.3 Graph demonstrating reasons for patients undergoing infertility treatment in 2008 in the UK HFEA (2010). Data representing male factor infertility as the most common cause of infertility.

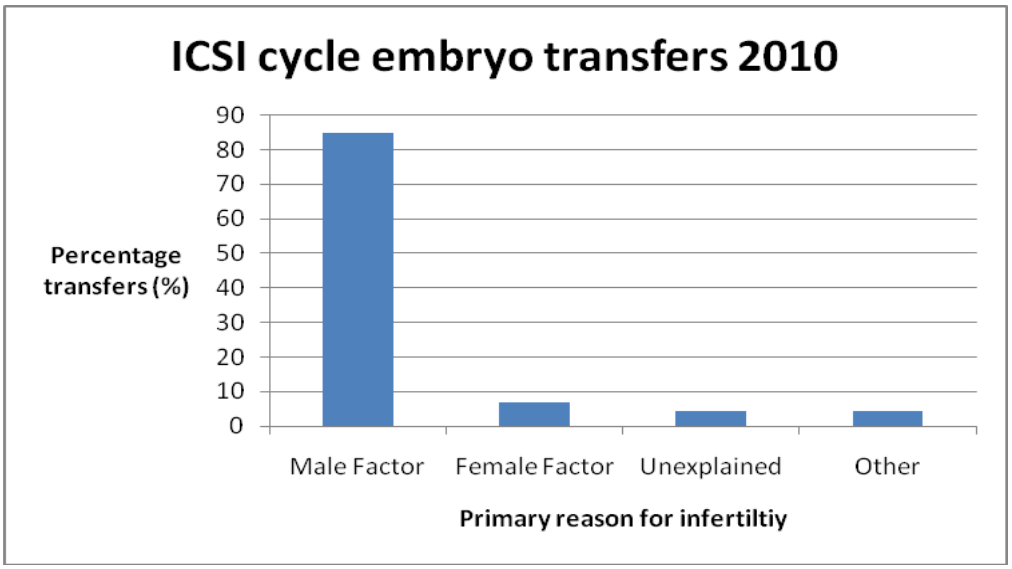

Figure 1.4 Interpretation of data from ACU Ninewells Hospital Dundee. Graph demonstrates the percentage of transfers from ICSI cycles by reason for infertility. Male factor includes various sperm abnormalities/failed vasectomy reversal, female factor includes tubal reasons/age/PCOS/anovulation/endometriosis, other includes previous failed fertilisation with IVF.

1.3 Sperm motility

Motility of a sperm cell is critical for fertility (Donnelly et al., 1998). In vivo, mammalian sperm must travel through the female reproductive tract to reach the site of fertilisation where fusion of male and female gametes takes place. In the female reproductive tract, sperm must accomplish the journey through a hostile, acidic environment, a barrier which is overcome by in vitro fertilisation. However, successful fertilisation in vitro is still dependent upon sperm motility (Simon and Lewis, 2011; Demir et al., 2011).

Sperm movement is generated by both the mid piece which consists of mitochondria to produce energy for sperm movement and the flagellum which propagates movement at the mid piece. The axoneme, a structure which runs through the sperm tail, moves in a way which causes flagellar waves propagating backwards to create forward propulsion (Mortimer, 1997). It consists of nine peripheral microtubule doublets and two central doublets which run the length of the flagellum and are protected by a cytoskeletal sheath (Inaba, 2003). Movement is achieved by the sliding of microtubule doublets by dynein arms (Mortimer, 2000). Dynein is responsible for converting chemical energy to mechanical energy from ATP hydrolysis for sperm motility. The sliding of microtubules creates bending and in turn, sperm motion.

Protein phosphorylation is involved in the initiation and regulation of flagellar motility. One way in which sperm motility is generated is through cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) signalling pathway. cAMP has long been known as a second messenger within cells and controls signalling pathways through the activation of protein kinase A (PKA) (Sutherland and Rall, 1960; Sutherland and Robinson, 1966; Sutherland et al., 1968). It is well established that cAMP and cGMP play an important role within human sperm involving motility and capacitation (Bajpai and Doncel, 2003). The level of cAMP within human sperm is therefore important regarding the fertilising potential of a sperm cell. A balance between inhibitory and stimulatory factors of cAMP is necessary to maintain the functional level. This balance exists due to the generating and degenerating processes of cAMP (Gagnon and De Lamirande, 2006). These

metabolic reactions include the synthesis of cAMP from adenosine triphosphate (ATP) mainly through soluble adenylate cyclase (sAC) and degradation by phosphodiesterases (PDEs) (Dessauer, 2009). The level of cAMP is therefore primarily controlled by the two enzymes sAC and PDEs along with external factors such as bicarbonate. sAC plays an essential role in the regulation of cAMP as without it, sperm are immotile and incapable of undergoing fertilisation (Esposito et al., 2004). Motility is generated through the activation of PKA by cAMP which is found within human sperm to phosphorylate substrate proteins giving rise to flagellar motility (Suarez, 2008). They are primarily involved in capacitation, the acrosome reaction and sperm function such as sperm-zona pellucida binding (Visconti and Kopf, 1998). It is therefore important that a balance exists in the level of cAMP to prevent premature hyperactivation, capacitation or acrosome reaction.

1.3.1 Inhibition of sperm motility

Contrary to this is the role of PDEs in human sperm. PDEs are known to breakdown cAMP. If too much cAMP is degraded, sperm motility may be interrupted, leaving sperm unable to function effectively. There are currently 11 different PDE gene families (Ghosh et al., 2009). PDEs are important regulators of signal transduction which degrade cyclic nucleotides i.e. the breakdown of the phosphodiester bond in cAMP and cGMP molecules. Both molecules are important in many biological processes, particularly the role of cAMP in mammalian sperm motility. It is well established that PDEs inhibit motility of human spermatozoa (Beavo, 1995).

Intracellular cAMP concentrations are regulated by stimulatory effects due to the permeability of the sperm cell. Stimulatory factors such as Ca^{2+} or HCO_3^- regulate these levels (Esposito et al., 2004). Changes in the level of cAMP, modulates activity of PKA resulting in a change of sperm motility. As cAMP is degraded it is converted into 5'AMP by PDEs (Figure 1.5). However, when produced by sAC, cAMP goes on to activate PKA which in turn activate PDEs. Functional levels of cAMP for sperm motility are not yet known however it is known that cellular functions within sperm are ultimately regulated by cAMP (Visconti et al., 1995). Subsequently, it is important that equilibrium between cAMP synthesis and degradation exists. Otherwise, the level of cAMP necessary for sperm motility may fluctuate. Consequentially, this may alter normal sperm function and in turn reduce the chance of a successful fertilisation.

1.3.2 Stimulation of motility – interfering with cAMP using PDE inhibitors

Since the early 1960s it was suggested that cAMP levels can be modulated by PDE inhibitors (Butcher and Sutherland, 1962). By the late 1960s it was known that the level of cAMP in sperm was important however the concept of enhancing cAMP using PDE inhibitors was only just being explored. A study by Yovich et al 1990 showed a strong correlation between Pentoxyfilline (PTX), a non-specific PDE inhibitor and fertilisation rates. This was a clinical study carried out on 57 couples with 51 having both known male factor infertility and previous failed IVF attempts. There was a significant ($P<0.001$) increase in fertilisation rate of 47.4% compared to

20.4% when sperm were incubated with PTX. This study demonstrated a beneficial effect of PDE inhibitors on human IVF success rates. Following this, another landmark study was carried out in 1992 by Tesarik et al using PTX and caffeine, (caffeine is another non-specific PDE inhibitor) to enhance sperm motility. This study explored the risk of acrosome reaction induction when 22 sperm samples were incubated with PDE inhibitors separately. The acrosome reaction is an important step in sperm-oocyte interaction and if prematurely induced, the sperm may not bind to the oocyte (Buffone et al., 2008). The fertilising ability of sperm decreases when the acrosome reaction has taken place prior to oocyte penetration. This study demonstrated the addition of PTX has no effect on the rate of acrosome reaction. However, caffeine significantly increased the rate of induced acrosome reaction (using calcium ionophore A23187) after 1 hour addition. Inducing the acrosome reaction raised concerns about the efficiency of PDE inhibitors and the concept of inhibiting, rather than promoting fertilisation. In 1993 Tasdemir et al also showed that PTX added to a total of 11 patient samples with previous failed or low fertilisation prepared by the swim up method enhanced the acrosome reaction. The inhibitor at a final concentration of 2mg/ml was added along with the acrosome reaction inducer calcium ionophore (A23187) to observe any enhancement on induced reactions. Although in the previous study discussed, induction of the acrosome reaction is suggested as a negative factor on the fertilising ability of human sperm, this study showed a strong correlation between samples pre-treated with PTX and pregnancy rates. Group A consisted of those with previous successful fertilisation having a pregnancy rate of 27.3% when treated with PTX, a significant increase in IVF rate ($P<0.001$). Group B which consisted of those with previous

failed fertilisation, also had a significant increase in IVF rate ($P<0.001$) with a pregnancy rate of 45.4% when treated with PTX. From this study it may be said that PTX improves fertilisation, especially in cases with previous fertilisation failure.

Tournaye et al 1993 however did not demonstrate a significant difference in fertilisation rates between groups of control and treated with PTX. This study included a total of 22 couples with male factor infertility and at least one previous IVF attempt resulting in failed fertilisation. No differences were noted in fertilisation rates between control and treated groups suggesting PTX does not benefit this group of patients. Notably, the sperm samples in this study were not prepared in the same way as studies such as Yovich et al 1990 where PTX was added after the wash rather than before in the case of this study. It is therefore possible that by washing sperm after the addition of a PDE inhibitor, its enhancing effect is lost and as a result, no improvement in fertilisation rates.

Negri et al 1996 however used PTX during IUI sperm preparation in a group of 101 cycles based on male factor infertility including asthenozoospermic and oligoasthenozoospermic patients. Patients used in this study had samples of normal morphology. The significance of incubating patient sperm with PTX was expressed by a rise in pregnancy rates ($P<0.05$). The standard preparation of sperm gave a pregnancy rate of 11.5% compared to 27.5% with samples treated with PTX out of 61 and 40 cycles respectively. The results suggest PTX improves the motility of sperm. This is validated since the group treated with IUI for other reasons (including

anovulation and endometriosis i.e. not male factor infertility) did not show any improvement in pregnancy rates when samples were treated with PTX.

The results of improving sperm for IUI are supported by a retrospective analysis study on 9963 IUI cycles (Stone et al., 1999). The aim of this study was to determine factors which influence the effectiveness of IUI including various methods of sperm sample preparation. One means of sperm preparation included the use of PTX as a motility enhancer demonstrating a higher overall pregnancy rate of 17.1% when sperm samples were suspended in culture media containing a final concentration of 3mmol/L PTX before Percoll processing. This is compared to 11% without the addition of any PDE inhibitor. Previous studies have included relatively small study groups whereas this study involved statistical analysis on a much larger scale and would therefore be more reliable. However, due to this and the exploration of different techniques of sperm preparation, this study is complex and does not concentrate specifically on the detail of PDE inhibitor addition.

Recent studies have demonstrated positive results using PTX. Mangoli et al 2011, used PTX for viability testing on non-motile sperm from testicular biopsies. This study compared PTX to the Hypo-osmotic swelling test (HOS), a well known viability test. They found an increase in pregnancy rates and fertilisation rates using PTX (32% and 62.05% respectively) compared to HOS test (16% and 41.07% respectively) to identify viable sperm. The HOS test has however been extensively studied and is known to be an easy, inexpensive and reliable method of sperm viability testing (Tartagni et al., 2004). This questions the need for another viability

test. However, recent studies have shown that PTX improves sperm motility (Kovacic et al., 2006). This study analysed 77 cycles of testicular sperm extraction (with ICSI) split into control group of 30 (without PTX) and treated group of 47 (sperm treated with PTX for 20 minutes). Immotile sperm from testicular samples became motile with exposure to PTX (1.76mM) for only 20 minutes. Fertilisation and pregnancy rates were higher for PTX treated cycles (66% and 38.3% respectively) than the control group (50.9% and 26.7%). Based on these results the authors concluded that PTX facilitates easy detection of viable sperm for use in ICSI. These studies are significantly important as it is known that the use of non-motile sperm increases the incidence of mitotic spindle defects (Tasdemir et al., 1998), which may be damaging to any resulting embryos or children born. However the subject of sperm viability testing prior to ICSI procedure is controversial due to the risk of damaging the limited sperm available. In response to the latest study by Mangoli et al 2011, Malpari et al state that there is no need to risk damage using PTX since sperm from biopsies are always viable even if immotile. This is because the testes only produce live cells and therefore no need to subject sperm to any testing which could potentially damage the cells. However, if this was the case it would be reasonable to assume all sperm would have fertilised mature oocytes (Kovacic, 2006).

Whilst studies both old and new have shown PTX amongst other PDE inhibitors have a significant effect on sperm motility, these inhibitors are not specific to certain individual PDEs and may inhibit more than one PDE type. Although sperm motility may be enhanced, any side effects of adding PDE inhibitors which are non-specific

remain unclear. Sildenafil is a PDE inhibitor which is known to be specific to PDE-5 (Corbin et al., 2003). It is commonly known as Viagra and in inhibiting PDE-5, increases levels of cGMP. A study carried out by Glenn, 2009, investigated the effect of using sildenafil in mice to determine fertilisation and embryo development. Male mice were fed sildenafil and allowed to mate with females injected with human chorionic gonadotropin (hCG). After female mice were killed their oviducts were dissected and embryos retrieved for assessment. They found a significant ($P<0.001$) decrease in fertilisation on day 1 by 33% where the male had taken sildenafil. They also found fewer embryos developed from all 4 days of killing where the male had taken sildenafil. For example, out of 21 mice killed without sildenafil the mean number of embryos was 23.1(+/-6.1) compared to 15.9(+/-4.8) with sildenafil, both goups killed on day 4, statistics are not given here. This study indicates a detrimental effect with the use of PDE inhibitors. Reasons for this may include alterations to sperm function such as the acrosome or the cause of sperm or embryo DNA damage (some of which will be explored in this study) or the inability of sperm/embryos to repair DNA damage. The fact that the number of motile sperm increases with PDE inhibitors along with the increased velocity would suggest more sperm reach the oocyte and therefore more chance of fertilisation. PDE inhibitors may counteract the positive effect on sperm motility by altering normal sperm function such as binding to or penetrating the oocyte. Recent studies have investigated the effect of PDE specific inhibitors on sperm motility. However, the use of PDE inhibitors on human sperm is controversial due to the limited amount of data available. From the 11 PDE families recognised to date, each have different isoforms. Individual PDEs differ in their type of regulation, sensitivity and localisation. Many types of PDEs can be

expressed in a single cell type which may also be the main regulators of cAMP and cGMP within a cell. This regulation can occur as biochemical interactions such as binding of Ca^{2+}-calmodulin (Ca^{2+}/CaM) and other protein interactions. Inhibitors control the temporal duration of cyclic nucleotides in the cell ensuring a local supply for the cell to function efficiently and at the same time not too much that will overload the cell (Houslay, 2010). The problem with PDE inhibitor control however is non specificity of some PDE inhibitors. Early studies have suggested that nearly all PDE inhibitors would inhibit most PDE types in a cell. Recent research however has recognised PDE inhibitors which are specific to a PDE type and should only influence the activity of that PDE (Bender and Beavo, 2006). It is for this reason PDE inhibitors have been used clinically. There is also belief that PDE type specific inhibitors can be developed to selectively target specific isoforms. The value of this is to avoid any occurring side effects that a non-specific inhibitor could cause. However due to limited data regarding type specific PDE inhibitors, little is known about the localisation and activity of PDEs within human sperm.

PDEs recognised in human sperm include PDE-1, 3, 4, 5 (Lefievre et al., 2002; Fisch et al., 1998; Cuadra et al., 2000). PDE-1 and 4 were some of the first PDE types suggested to be present in human sperm through the use of PDE type specific inhibitors. Fisch et al 1998, demonstrated that an inhibitor of Ca^{2+}/CaM regulated PDE-1, 8-methoxy-isobutylmethylxanthine (8-MeOM-IBMX) at a final concentration of 100µM and cAMP-specific PDE-4, RS-25344 (Rolipram) at a final concentration of 10 µM had significant effects on human sperm activity. Firstly they showed that 35-40% and 25-30% of PDE activity was 8-MeOM-IBMX and

Rolipram sensitive respectively suggesting PDE-1 and 4 are present in human sperm. Their data also demonstrates that 95% of PDE activity is IBMX sensitive which supports the fact that IBMX is non-specific compared to PDE-1 and 4 inhibitors which are selective. They then demonstrated that sperm motility can be enhanced using Rolipram on 27 healthy donor sperm samples after a 2 hour incubation period. This result was significant ($P<0.0001$) even in the donor population as the average motility increased from 71.1%(+/-1.9) in the control group compared to 76.1%(+/-1.3) in the treated group. A significant ($P=0.00008$) increase in sperm motility was also observed using 12 abnormal samples with average motility increasing from 65.6%(+/-2.0) to 74.3%(+/-1.5) in the treated group. There was however no significant change in motility using 8-MeOM-IBMX on either sperm sample group. When investigating the effect of PDE inhibitor on the acrosome reaction they observed 8-MeOM-IBMX selectively stimulated the acrosome reaction whereas Rolipram did not. This evidence supports the hypothesis that PDE subtypes do exist within human sperm and inhibiting them may affect sperm activity.

PDE-1s were one of the first of the PDE family to be located within the human sperm cell (Lefievre et al., 2002). PDE-1A (an isoform of PDE-1) was localized on the equatorial segment of the sperm head as well as on the mid and principal pieces of the flagellum by immunocytochemistry experiments using isoform specific antibodies. PDE-3A (an isoform of PDE-3) was also located on the post acrosomal segment of the sperm head. However, there is limited information on the effect of inhibition of specific PDEs within human sperm.

Recent development in PDE targeting has been investigated in other cell types (Witzenrath et al., 2009; Rahrmann et al., 2009). PDE inhibitors are commonly used drugs with various uses in medicine. They are already used clinically for a range of diseases and disorders and have been proven safe for use. Type specificity is of crucial importance to eliminate the possibility of other PDEs being inhibited and in turn causing detrimental effects. PDE inhibitors are commonly used as therapeutic solutions to a variety of disorders. They range from cognitive disorders such as schizophrenia and dementia (Zhang, 2010) to inflammatory disorders such as asthma and bladder inflammation (Kitta et al., 2008). Others include the PDE-5 inhibitor commercially known as sildenafil (as discussed previously), which increases the availability of cGMP in the cell, and is not only used as an erectile dysfunction drug but may also be a possible solution to heart failure (Guazzi et al., 2010). Others specific to their corresponding PDEs include BRL, specific to PDE-7 and Papaverine specific to PDE-10. BRL is used in airway and immunological applications since PDE-7 has been expressed in human proinflammatory and immune cells (Smith et al., 2004). This study also demonstrated that BRL failed to inhibit other PDEs such as PDE-1, PDE-2 and PDE-5, thus providing encouraging results for the clinical use of this inhibitor. Papaverine is used for schizophrenia and other neurological disorders (Ghosh et al., 2009). This PDE inhibitor is reported to be specific to PDE-10 and related to Huntington's Disease (Hebba et al., 2004).

Recent advances in the field of PDE research have rekindled interest in the clinical advantage of PDE inhibitors. However, even with positive results it is surprising that little is known about the effect of type specific inhibitors on sperm; particularly since

non-specific inhibitor studies have shown to have a positive effect on sperm motility. However, there is no information regarding the effect of PDE inhibitors on human sperm function at the sperm-oocyte interaction stage. Although, PDE inhibitors are reported to have a positive effect on sperm motility it is important that this is maintained throughout the stages of fertilisation. It is also important that PDE inhibitors do not cause any detrimental effects to the sperm-oocyte interaction or the development of any resulting embryos.

1.4 Sperm-oocyte interaction

Although type specific PDE inhibitors have been used to influence the motility of a sperm cell, they have never been used to test sperm function such as the interaction between sperm and oocyte including penetration of the cumulus, binding to the zona pellucida and fusing with the oolema. Since sperm motility can be enhanced using PDE inhibitors there is a possibility that sperm function may be restored i.e. such as the ability to acrosome react or bind to the zona pellucida. Enhancing or even restoring the function of defective sperm could result in an increase of ICSI to IVF (or even further to IUI) conversions for patients. Thus reducing treatment cost for the patient, invasiveness of the oocyte (or patient in the case of IUI) and an embryologists time required to carry out the ICSI procedure. Improving sperm function at the sperm-oocyte interaction stage suggests an increase in fertilisation and pregnancy rates for ART clinics since firm attachment of gametes has previously predicted IVF outcome (Liu et al., 2011). However, there is no information regarding

the effect of PDE inhibitors on human sperm-oocyte interaction, hence no correlation between PDE inhibitors and sperm function at this critical stage.

Motility is known as one of the key predictors of fertilisation outcome as it predicts cumulus and zona pellucida penetration by the sperm cell (Oehninger, 1992). The interaction between sperm and oocyte is a critical event leading to fertilisation. Multiple sperm functions are reflected by this step including the ability of the sperm to bind to the zona pellucida, undergo acrosome reaction and fuse to the oolema (Suarez et al., 2006). Motility is also important for sperm to generate propulsive forces which are necessary to penetrate the zona pellucida (Mortimer, 1997). A way of predicting the effect of PDE inhibitors on penetration and overall fertilisation would be investigating the ability of sperm to bind to the zona pellucida. Sperm-zona pellucida binding is arguably the most important stage of fertilisation and has been investigated since the late 1980s (Franken et al., 1989). Two types of sperm-zona pellucida binding tests have previously been used as predictors of fertilisation in vitro including the hemizona assay (HZA) and sperm-zona pellucida binding test. These studies have shown a significant correlation with fertilisation rates providing a diagnostic tool for patients undergoing IVF treatment (Franken et al., 1989). Following this, recent studies have also suggested the use of zona pellucida bound sperm for treatment (Liu et al., 2011). Other studies have suggested the use of sperm-zona pellucida binding as a diagnostic tool in order to predict and prevent failed fertilisation (Lee et al., 2008). Some have even suggested that the sperm-zona pellucida method has the potential as a contraceptive intervention (Dun et al., 2010). Overall, the sperm-zona pellucida binding test is viewed as a reliable and useful

method for determining the quality and ability of a sperm cell. Investigating sperm function at the fertilisation level by the inhibition of PDEs is a major step in the advances of ART and may provide useful clinical information. PDE inhibitors are therefore a growing interest as targets for therapeutic intervention.

1.5 Aims and objectives

The principle aim of this study was to investigate the effect of PDE inhibitors on human sperm motility and function. Experiments were carried out using CASA and various sperm function tests with a variety of PDE inhibitors. ART patients were used in this study to determine whether PDE inhibitors have any clinical beneficial effects. The aim of this was to provide a better understanding of the effect of PDE inhibitors on patient sperm, in turn providing a clinical insight into the effect of PDE inhibitors.

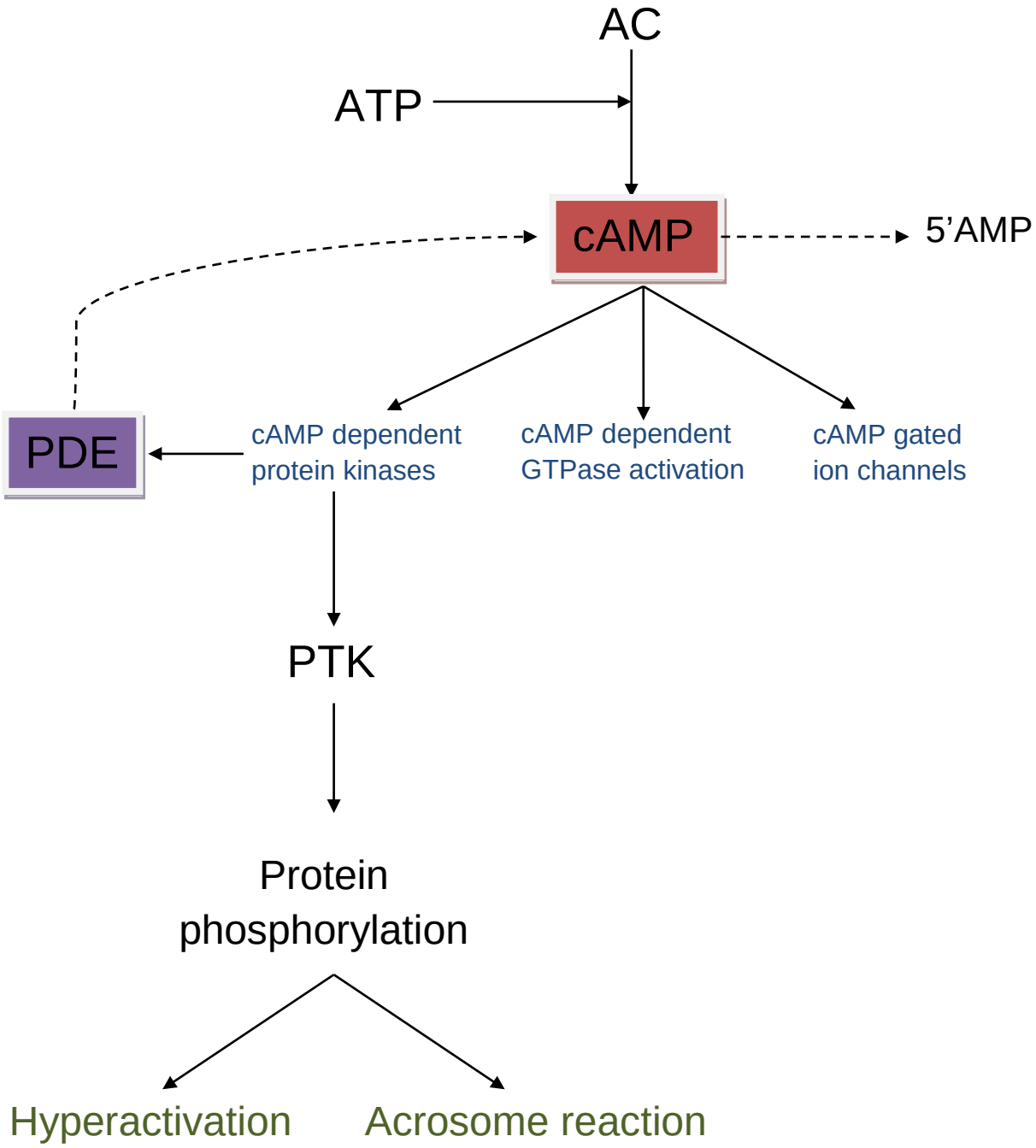

Figure 1.5 Personal interpretation of cAMP dependent pathway. Figure demonstrates the action of PDE in the synthesis of cAMP to 5'AMP.

Chapter 2: Investigating the effect of PDE-1 and PDE-4 specific inhibitors on human sperm motility

2.1 Introduction

PDE-1 and PDE-4 have been studied extensively in various cell types. However, not many studies have investigated the existence and function of these PDEs in sperm. Few studies have examined PDE-1 and PDE-4 in sperm with their corresponding inhibitors 8-MeOM-IBMX and Rolipram respectively.

PDE-1 was one of the first PDE families to be identified and has been extensively studied in other cell types. PDE-1 has three different isoforms, PDE-1A, PDE-1B and PDE-1C. PDE-1 is regulated by Ca^{2+}/CaM which stimulates cyclic nucleotide hydrolysis. The PDE-1 enzymes can hydrolyse both cAMP and cGMP however the affinity for each nucleotide varies depending on which isoform. PDE-1 isoforms have been expressed in the brain and peripheral neurons, the heart and cardiovascular vessels and the macrophages and T lymphocytes of the immune system (Medina, 2011; Movsesian and Kukreja, 2011; Kanda and Watanabe, 2001). More relevant to this study however, PDE-1 is has been located in the human testis (Fidock et al., 2002) and in sperm with most activity found in the mid-piece and tail (Lefievre et al., 2002). Previous studies demonstrated the presence of PDE-1A and PDE-1C using immunocytochemistry in germ cells in the mouse testis (Yan et al., 2001). These PDE forms were highly expressed in developing and mature sperm suggesting PDE-1 has a role in spermatogenesis with the expression of PDE-1 forms relating to the

different stages in development. In 2002, Lefievre et al demonstrated the presence of PDE-1A along with PDE-3A in the equatorial section and flagellum, and sperm head respectively of human sperm. These results gained through immunoblotting, confirmed the presence of PDE-1 isoforms in human sperm. This group go on to suggest PDE-1A is permanently active in sperm since Ca^{2+}/CaM is tightly bound to PDE-1A and cannot be dissociated. The presence of more than one PDE-1 isoform was confirmed in 2005 as Vasta et al identified a new variant of PDE-1A in mouse sperm using immunocytochemical staining. PDE-1A revealed intense expression in the sperm tail. These results suggest that more than one PDE isoform is active in sperm and a step further to understanding the role of PDEs in regulating sperm function.

PDE-4 is expressed in various cell types and plays major roles in many biological processes. There are four genes PDE-4A, PDE-4B, PDE-4C and PDE-4D that make up the PDE-4 family (Bender and Beavo, 2006). PDE-4 is one of the most studied PDEs in various cell types with extensive information available on its physiological function. PDE-4 is recognised to have cAMP specific activity which is characterised when inhibited by the PDE-4 selective inhibitor known as Rolipram. PDE-4 has been associated with various diseases such as heart disease, since cAMP is a regulator of cardiac contractility. Studies using PDE knock-out mice give a valuable insight into the importance of PDEs. For example, PDE-4D deficient mice have progression of heart failure (Lehnart et al., 2005). Jin and Conti in 2002 investigated PDE-4B knock-out mice which demonstrated the importance in immune cells as PDE-4B deficient mice did not show an inflammatory response to lipopolysaccharide in

monocytes. These studies clearly indicate individual PDEs are related to different functions and emphasises the importance for type specific inhibitors if used clinically. The evidence of PDE-4 in sperm however is very limited. Some studies have investigated the effect of PDE-4 inhibitor Rolipram (Fisch et al., 1998). However, there appears to be no recent development or further investigation. This section investigates the effect of 8-MeOM-IBMX and Rolipram on the percent of motile cells of donor and patient sperm samples.

2.2 Materials and Methods

Experiments were carried out on a pool of either sperm donors with no known fertility problems or sperm samples from patients undergoing IVF/ICSI. The mean percent of motile cells was the focus in this study. After density gradient centrifugation, both the 40% and 80% fractions were used from donor samples and only the 40% fraction used from patient samples as the 80% was not available at this time. The 80% pellet contains the more dense mature sperm with a higher percentage of progressively motile cells whereas the 40% is known to contain the less motile, immature sperm (Kessopoulou et al., 1992). The difference between the separate fractions with regards to the percentage of motile sperm has been correlated with fertilisation and pregnancy rates (Donnelly et al., 1998). In this study both fractions were investigated where possible to gain a better understanding of the contrast of motility within different fractions and the true effect of PDE inhibitors.

2.2.1 Sperm Preparation

2.2.1.1 Donor Sperm Preparation

Semen samples were obtained from three consented 'healthy' donors. Samples were produced at home after 2-5 days of sexual abstinence and collected in sterile 60ml containers. Samples were obtained within 1 hour of production and incubated at 37°C until liquefied. An initial assessment of sperm concentration and motility was carried out in order to investigate the quality of the individual samples using CASA (Computer Assisted Sperm Analysis) by placing 4µl of the raw sample on a 20µm depth Hamilton-Thorne slide with a 22mm by 22mm coverslip. Up to 2mls (where possible, dependant on sample volume) of semen sample was then layered on a PureSperm® gradient of 40% and 80% in a non-sterile 15ml conical bottom falcon tube. Centrifugation was carried out at 300g for 20 minutes. The supernatant was discarded and either the 40% fraction or the pellet was then taken by a Pasteur pipette and placed into a non-sterile 15ml conical bottom falcon tube containing 5mls HEPES buffered Earle's medium (1.8mM $CaCl_2$, 5.37mM KCl, 0.81mM $MgSO_4$, 1.01mM NaH_2PO_4, 116.36mM NaCl, 5.55mM D-Glucose, 2.73mM $C_3H_3NaO_3$, 41.75mM $NaC_3H_5O_3$, 25mM HEPES pH 7.4). Each fraction was centrifuged at 500g for 10 minutes allowing the sperm to be 'washed', removing any remaining PureSperm®. Pellets from both fractions were suspended in 500µl pre-equilibrated synthetic tubal fluid (STF) medium (3.0mM $CaCl_2$, 4.7mM KCl, 1.0mM $MgSO_4$, 106.0mM NaCl, 1.5mM NaH_2PO_4, 5.6mM D-Glucose, 1.0mM $C_3H_3NaO_3$, 41.8mM $C_3H_5NaO_3$, 1.33mM Glycine, 0.68mM Glutamine, 0.07mM Taurine, 25mM

$NaHCO_3$, pH 7.4 supplemented with 30mg/ml human serum albumin). The fractions were then incubated at 37°C with 6% CO_2 for at least 3 hours in order for the samples to capacitate. Sperm concentration and motility were assessed from both the 40% and 80% suspensions using CASA and a dilution made to the required sperm concentration of approximately $20x10^6$/ml. Sperm morphology was not carried out in this study. Both 40% and 80% fractions were assessed.

2.2.1.2 Patient Sperm Preparation

Samples were obtained from three Ninewells Hospital Assisted Conception Unit (ACU) patients consented for this study. Patient samples were produced in the ACU and allowed to liquefy in a 37°C incubator within 30 minutes allowing the sperm to become freely motile. Sperm preparation for IVF/ICSI treatment was carried out in the ACU by an embryologist and any surplus of raw sample was used in this study. An initial assessment of sperm concentration and motility were carried out to investigate the quality of the individual samples using CASA as above. The samples were prepared as above however only the 40% fraction was used from patient samples. The pellets were suspended as above in media depending on whether they were IVF or ICSI. IVF samples were suspended in pre-equilibrated 500µl synthetic tubal fluid (STF) medium and ICSI samples in HEPES buffered Earle's medium. IVF samples were then incubated at 37°C with 6% CO_2 for at least 3 hours in order for the samples to capacitate whereas ICSI samples were left at room temperature. Sperm concentration and motility were assessed from the suspensions and a dilution

made to the required sperm concentration of approximately $20x10^6$/ml in their corresponding media. Sperm morphology was not included in this study. Only the 40% fraction was assessed.

2.2.2 Sperm-PDE inhibitor incubation

Following sperm preparation and capacitation (see 2.2.1.1 and 2.2.1.2), 99µl of each sample was placed into 1.5ml eppendorfs of control and treated. The treated labelled eppendorf was then supplemented with 1µl of a selected PDE inhibitor (8-MeOM-IBMX and Rolipram used in this section) making up to a required final concentration. PDE inhibitor concentrations were selected according to previous literature (see table 2.1). Inhibitors 8-MeOM-IBMX, Milrinone and Rolipram were used at concentrations previously studied whereas the concentration of Papaverine and BRL (PDEs which have not been explored in human sperm) was used as a starting point to gain an insight into whether there is any effect of these inhibitors on human sperm motility. Eppendorf lids were pierced to allow entry of CO_2. PDE-1 and 4 specific inhibitors (8-MeOM-IBMX and Rolipram respectively) were used at concentrations of 100µM and 10µM respectively (Fisch et al., 1998). Table 2.1 demonstrates the inhibitors selected for use throughout the study and the final concentrations at which they were used:

	PDE Type	**Final Concentration (µM)**
8-MeOM-IBMX	PDE-1	100
Milrinone	PDE-3	50
Rolipram	PDE-4	10
BRL	PDE-7	100
Papaverine	PDE-9	100

Table 2.1 PDE inhibitors and their corresponding final concentrations used in this study. Concentrations based on previous studies (Fisch et al., 1998; Lefievre et al., 2002).

Samples were incubated with a selected PDE inhibitor at 37°C, 6% CO_2 for a select time period (0.5, 1 and 2hr in this section). After gentle mixing, 4µl was taken from each aliquot (control and treated) for analysis and placed on a 20µm depth Hamilton-Thorne slide with a 22mm by 22mm coverslip after the slide was left on a heated stage for 1 minute, to allow temperature equilibration. The percent of motile sperm was assessed using CASA as this is one of the most significant predictors of fertility, alongside sperm concentration (Larsen et al., 2000). Parameters used to determine sperm characteristics pre and post PDE inhibitor addition are shown in table 2.2. ANOVA was used in statistical analysis to compare the mean of both control and treated over several incubation time points and P value <0.05 was considered significant.

Terminology	Definition
Static	No movement
Slow	<5 μms^{-1} VAP
Medium	5-25 μms^{-1} VAP
Rapid	>25 μms^{-1} VAP
Motile	Rapid + medium + Slow
Progressive motility	VAP > 25 μms^{-1} STR > 80%

Table 2.2 Standard CASA parameters used to analyse human sperm motility. Percent of motile sperm includes slow, medium and rapid cells. Progressively motile cells (as used in later chapter) includes those with an average path velocity (VAP) of greater than 25 µms-1 and a linearity of greater than 80%.

CASA

CASA is an automated digital system which can analyse motility characteristics and predict the fertility potential of sperm (Youn et al., 2011). Early generation CASA systems were only suitable for research purposes due to their inaccuracy in semen analysis (Mortimer and Mortimer, 1988), however the development of CASA has led to a clinical application. The advantages of CASA over manual semen analysis first include the accuracy of assessing subtle movement characteristics of sperm which cannot be done by eye including velocity. Secondly, CASA can be carried out in a matter of seconds, measuring hundreds of cells, again impossible to do manually. The use of CASA in this study was decided upon and used throughout due to these factors and for the purpose of standardisation.

How CASA works

In this study, a Hamilton Thorne 'CEROS' computer assisted sperm analyser was used with negative phase contrast and charged couple device (CCD) video camera showing white sperm against a dark background. This was combined with the clinical human application (Version 12). As the camera scans the image it combines fields to form a frame and in this study a frame rate of 60Hz was used to reconstruct an accurate measurement of sperm movement as recommended for the analysis of human sperm (Owen and Katz, 1993). Images from a microscope field are converted into a digital image by the camera from the size and illumination of sperm heads and a trajectory for each cell can be reconstructed (Mortimer, 1997). Although sperm motility largely depends on the flagellum, it is not possible to gain a clear image of this structure and so it is easier to analyse sperm head movement. Trajectories provide information on sperm motility and are one of the main advantages of CASA since manual trajectory reconstruction would be difficult to achieve. Included in this are three velocity parameters. Straight line velocity (VSL) is the distance between the start and the end of sperm trajectory, in other words, the distance covered in a straight line. The curvilinear velocity (VCL) is the measure of the overall distance travelled in any direction. The average path velocity (VAP) is the length of the general trajectory which is the distance of the average direction of movement. Values derived from the velocity parameters include the linearity ((VSL/VCL)x100) and straightness ((VSL/VAP)x100) which allow a more detailed analysis of movement and distinction between types of motility. Other parameters include the amplitude of

lateral head displacement (ALH) and beat cross frequency (BCF) which measure the movement of the sperm head from side to side and the flagellar beat respectively.

Relevant motility parameters

Since sperm move in ways that are adapted to their functional needs, the parameters chosen to represent the questions in this study include percent motile cells, percent progressively motile cells and the average path velocity. Firstly the percent of motile cells is an important element of IVF insemination and is defined by the total of rapid and medium cells, in other words with a speed of above 5µm/s (Table 2.2). Secondly, the percent of progressively motile cells which is important particularly for IUI where sperm have a greater distance to swim. This is defined by an average path velocity of greater than 25µm/s and a straightness of more than 80% which is calculated by the straight line velocity and average path velocity. The average path velocity is the velocity along the average path of the sperm and is another parameter which involves the progression of a sperm cell. Velocity is important for the penetration of cervical mucus and sperm-egg interaction.

Clinical application of CASA

Studies have shown the clinical potential of CASA with the relationship between sperm movement and fertilisation potential of both the general population and those with unexplained infertility (Larsen et al., 2000; Youn et al., 2011) and those specifically with male fertility problems (Aitken et al., 1984; Barratt et al., 1993; Paston et al., 1994; Krause, 1995). The various parameters of sperm motility allow the prediction of the function ability of sperm such as penetration of cervical mucus

or penetration of the zona pellucida, both important factors in fertilisation and both investigated in this study.

Although evidence suggests a link between the use of CASA and fertility, there is a lack of standardisation between laboratories. One of the major issues surrounding this is the definition of specific motility parameters such as hyperactivation which will not be investigated here (Mortimer, 1997). Others include the effects of sperm preparation technique and culture conditions between laboratories, making it difficult to compare studies. There are also various makes and models of CASA which have discrepancies between them. However, studies have still shown significance with the use of CASA and fertilising ability of sperm within their settings.

2.3 Results: Effect of PDE inhibitors on sperm motility

2.3.1 Effect of PDE inhibitors on 'healthy' donor population

There was no significant increase in the percent of motile cells in either 8-MeOM-IBMX or Rolipram treated 80% fractions of donor sperm (figures 2.1 and 2.2). The results at 0.5, 1 and 2 hours incubation between control and treated were not statistically significant ($P > 0.05$).

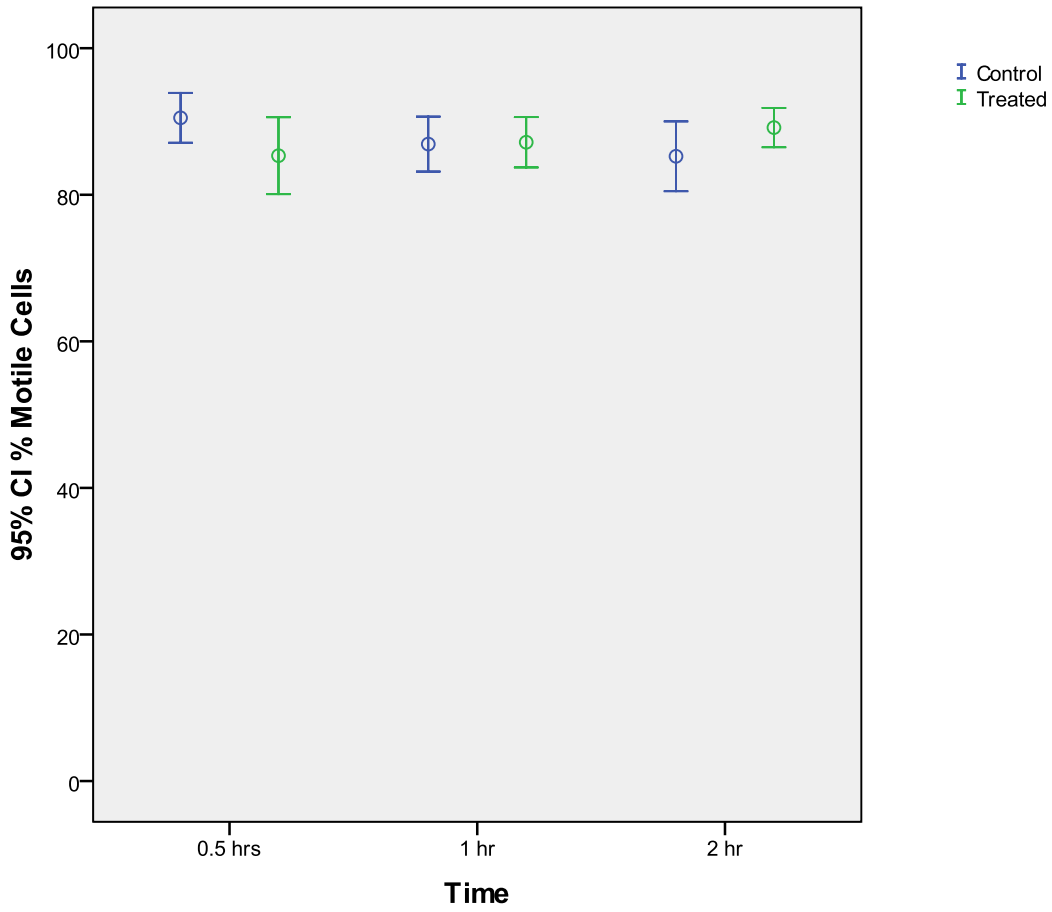

Figure 2.1 Each bar represents the mean percent of motile cells in the 80% fraction of 3 different donor sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of 8-MeOM-IBMX. P>0.05 therefore no statistical difference between the control and treated at any time point.

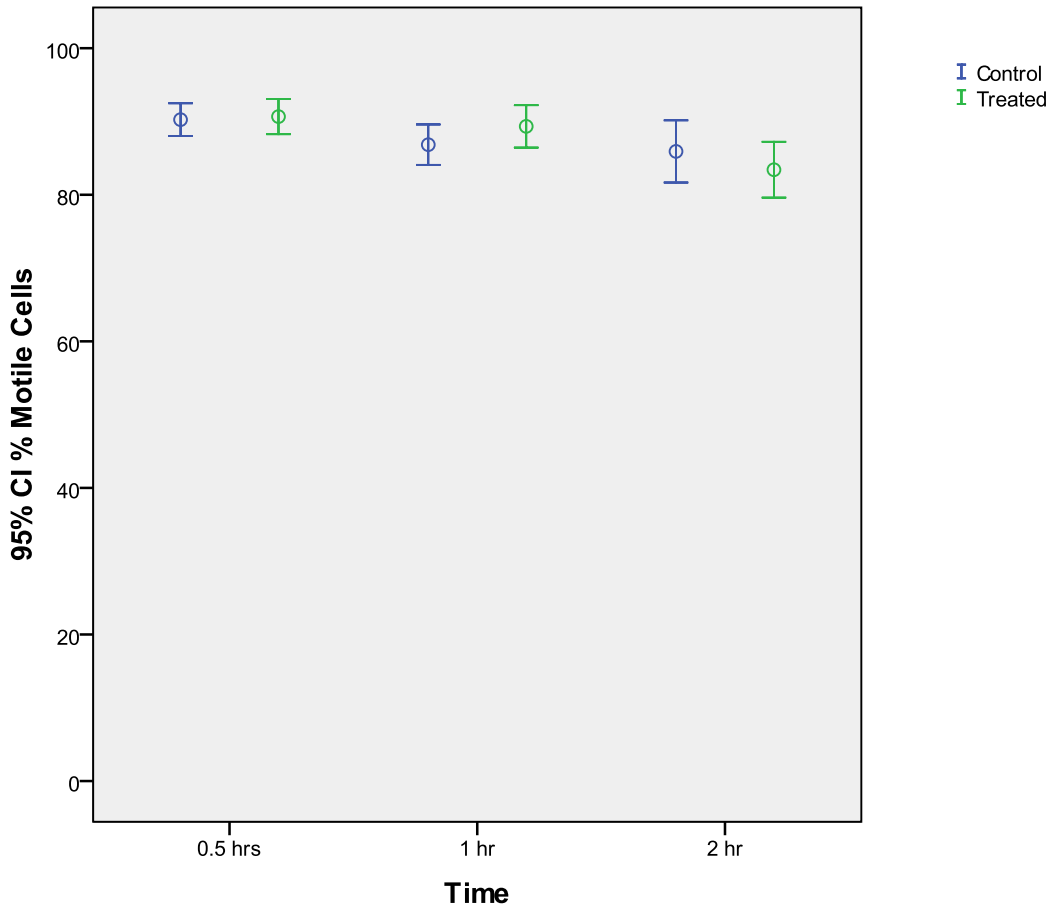

Figure 2.2 Each bar represents the mean percent of motile cells in the 80% fraction of 3 different donor sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of Rolipram. P>0.05 therefore no statistical difference between the control and treated at any time point.

The results also demonstrate no significant difference in the percent of motile cells when the 40% fraction of donor sperm is incubated with either 8-MeOM-IBMX or Rolipram at 0.5, 1 and 2 hour incubation periods (P > 0.05) (Figures 2.3 and 2.4).

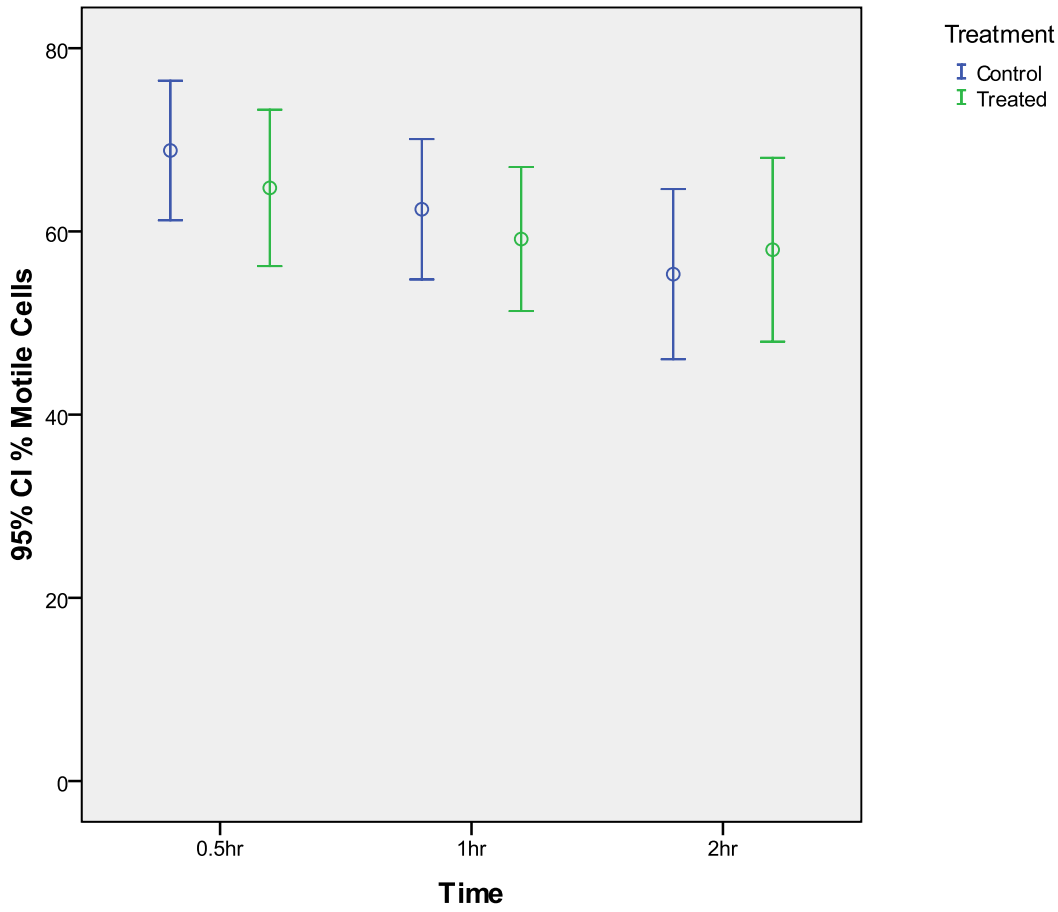

Figure 2.3 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different donor sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of 8-MeOM-IBMX. P>0.05 therefore no statistical difference between the control and treated at any time point.

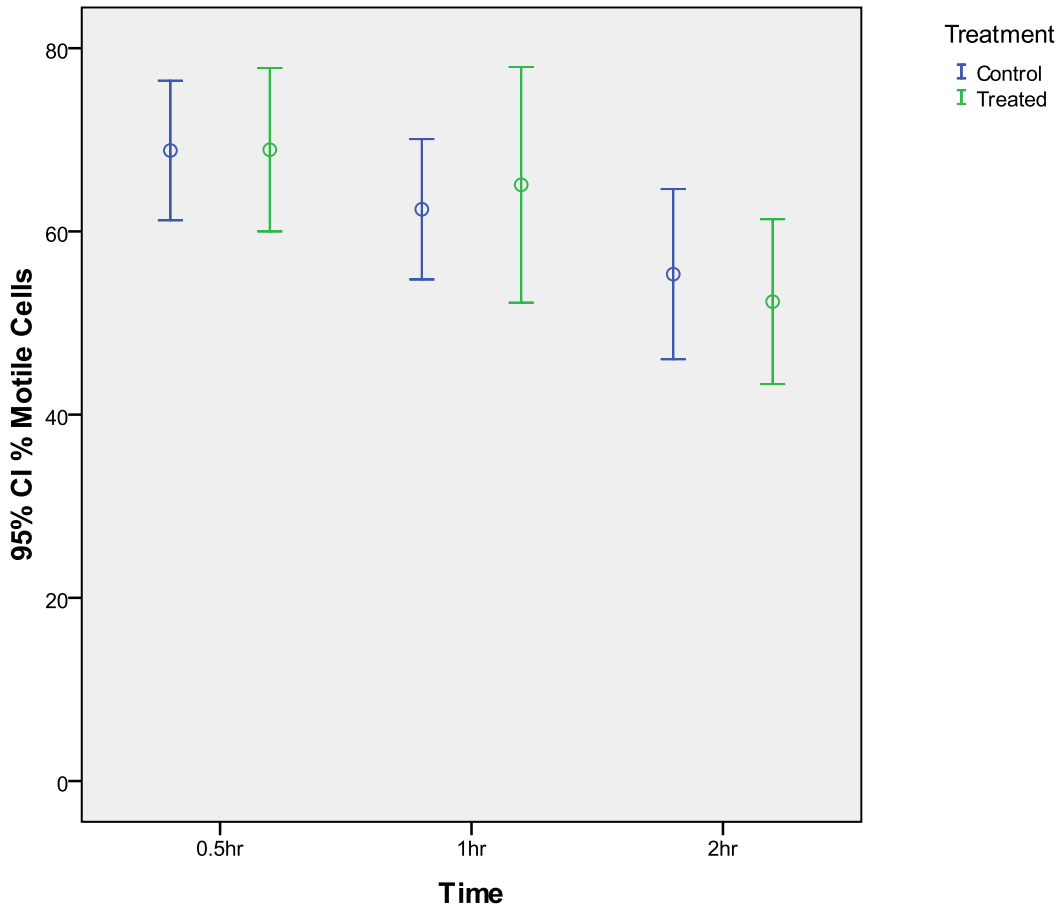

Figure 2.4 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different donor sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of Rolipram. P>0.05 therefore no statistical difference between the control and treated at any time point.

2.3.2 Effect of PDE inhibitors on patient samples

After 1 hour incubation with 8-MeOM-IBMX there was a significant increase in the percent of motile cells in the 40% fraction of three patient samples (Figure 2.5). There was also a significant increase in the percent of motile cells when the 40%

fraction of patient samples were treated with Rolipram at 0.5, 1 and 2 hour incubation periods (Figure 2.6).

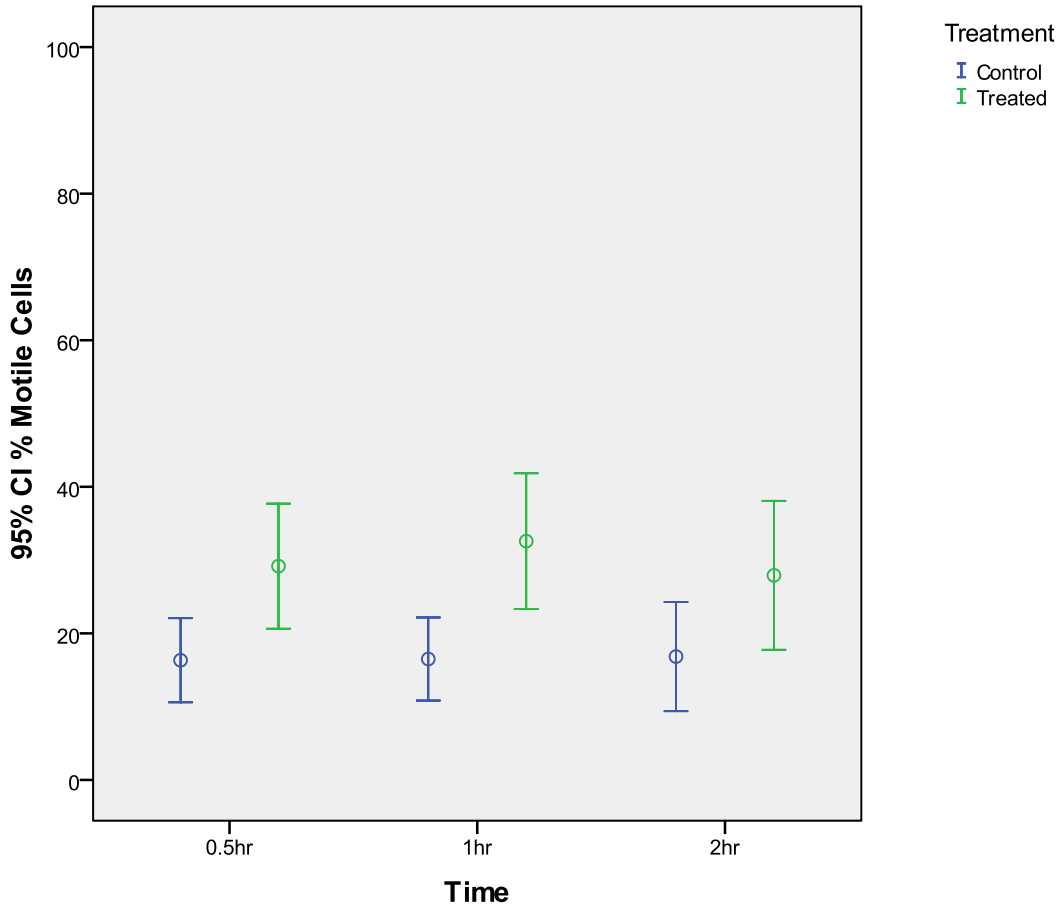

Figure 2.5 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different patient sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of 8-MeOM-IBMX. $P<0.05$ at 1 hour incubation time therefore a statistical difference between the control and treated.

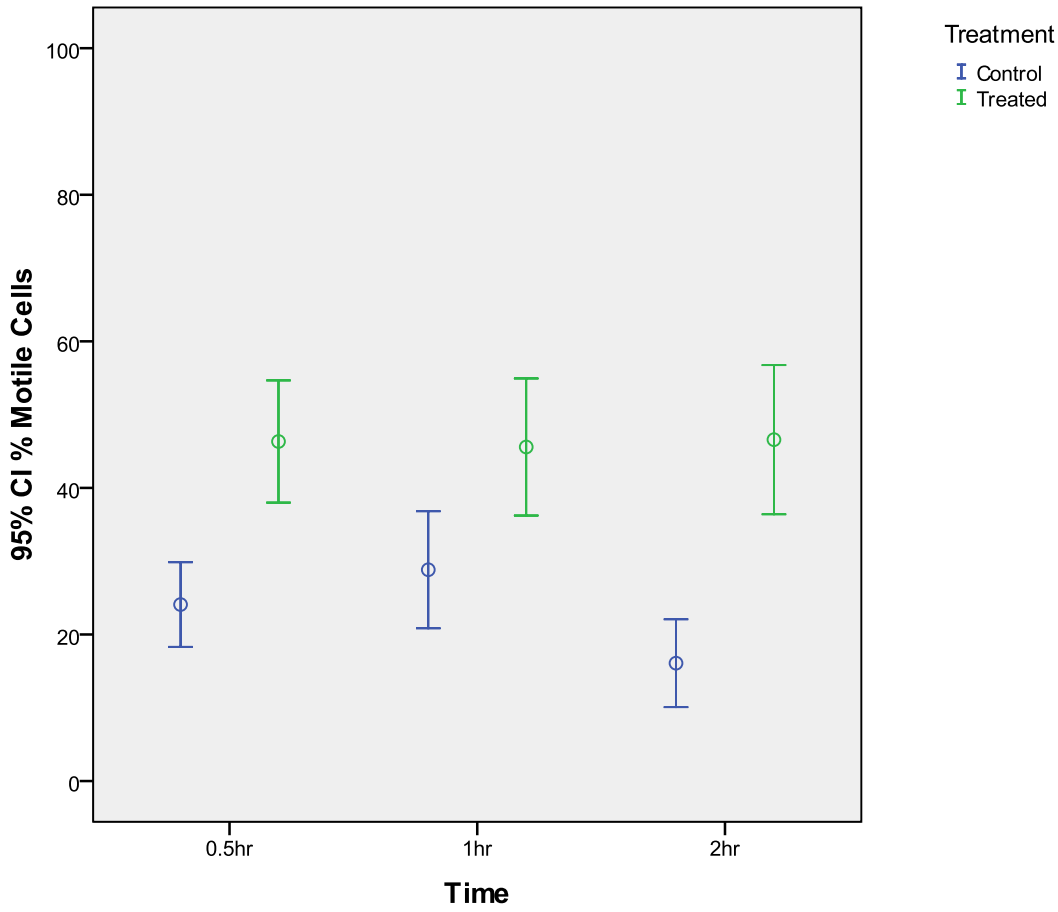

Figure 2.6 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different patient sperm samples at 0.5, 1 and 2 hours incubation with and without the addition of Rolipram. $P<0.05$ throughout incubation times and therefore a statistical difference between the control and treated.

The difference between the quality of 40% fractions of donor and patient sperm was clear with regards to motile sperm, even without the addition of PDE inhibitors. For example, after 1 hour incubation the mean percent of motile cells in the control population of donor sperm was 62.4% ± 6.1 compared to16.5% ± 9.0 for the control population of patient sperm (See tables 2.3 and 2.4 for further details). This table

demonstrates that the target population for improving motility is patient sperm samples.

Time (hours)		40% fraction treated with 8-MeOM-IBMX	Motility Mean % ± SEM	P-value
0.5	Donor	C T Difference	68.8 ± 10.7 64.8 ± 13.8 4 ± 3.1	0.47
	Patient	C T Difference	16.3 ± 8.6 29.2 ± 13.0 12.9 ± 4.4	0.2
1	Donor	C T Difference	62.4 ± 6.1 59.2 ± 3.9 3.2 ± 2.2	0.13
	Patient	C T Difference	16.5 ± 9.0 32.6 ± 13.4 16.1 ± 4.4	0.036
2	Donor	C T Difference	85.9 ± 6.7 83.8 ± 5.1 2.1 ± 1.6	0.28
	Patient	C T Difference	16.8 ± 9.6 27.9 ± 15.6 11.1 ± 6	0.37

Table 2.3 Effect of 8-MeOM-IBMX on the percent of motile cells in the 40% fraction of donor and patient human sperm samples. CASA performed at 0.5, 1 and 2 hours.

Time (hours)		**40% fraction treated with Rolipram**	**Motility Mean ± SEM**	**P-value**
0.5	Donor	C T Difference	68.8 ± 10.7 68.9 ± 13.4 0.1 ± 2.7	0.96
	Patient	C T Difference	24.1 ± 7.4 46.3 ± 2.8 22.2 ± 4.6	0.03
1	Donor	C T Difference	62.4 ± 6.1 65.1 ± 13.5 2.7 ± 7.4	0.61
	Patient	C T Difference	28.8 ± 11.3 45.6 ± 9.9 16.8 ± 1.4	0.02
2	Donor	C T Difference	55.3 ± 15.2 52.3 ± 15.3 3 ± 0.1	0.08
	Patient	C T Difference	16.1 ± 5.8 46.6 ± 1.2 30.5 ± 4.6	0.009

Table 2.4 Effect of Rolipram on the percent of motile cells in the 40% fraction of donor and patient human sperm samples. CASA performed at 0.5, 1 and 2 hours.

2.4 Discussion

PDE-1 and 4 inhibitions (8-MeOM-IBMX and Rolipram respectively) do not have any significant effect on the percent of motile cells of the 80% fraction of donor sperm samples. Reasons for this may be that sperm from a good quality sample cannot be improved. There was also no effect on the 40% fraction of donor sperm. The quality of a 40% fraction is generally poorer than that of an 80% fraction from sperm preparation and so it would be expected that the 40% fraction may be

improved (Mortimer, 2000). However, this was not the case with the samples used in this study. Reasons for this may be that the samples were of such good quality that even the 40% fraction was not as poor as expected. In contrast, patient samples were poor with regards to the percent of motile sperm and therefore were improved. The percent of motile cells was initially relatively low and increased with the addition of either 8-MeOM-IBMX or Rolipram in patient samples. The average number of motile cells shown in the graphs however was from three different patients of a mix of both IVF and ICSI. Due to the varying quality, IVF and ICSI samples are separated later in the study. In conclusion, PDE inhibitors 8-MeOM-IBMX and Rolipram increase the percent of motile cells in patient sperm samples. For this reason it is mainly patient samples which are used during the study.

Chapter 3: Investigating the effects of PDE inhibitors using different sperm media, PDE inhibitor concentrations and longer incubation periods

3.1 Investigating the effect of PDE inhibitors using non-capacitating media

3.1.1 Introduction

It is known that sperm motility is effected by preparation and motility can be provoked and maintained by capacitating conditions (Moseley et al., 2005). As shown in chapter 2, PDE inhibitors have a positive effect on the percent of motile cells in patient sperm samples. However, the method of sperm preparation varies between ART clinics including the technique and media used. Media used also depends on which procedure, for example in Dundee's ACU ICSI is carried out in non-capacitating conditions whereas IVF is carried out in sperm media and requries the effect of CO_2 for pH equilibration and sperm capacitation. Considering the variation between procedures and different units it is important to determine whether the effect of PDE inhibitors on patient sperm motility is maintained in non-capacitating media. Sperm samples suitable for Intra-uterine insemination (IUI) are also often prepared in non-capacitating media. Reasons for this include sperm for IUI are to be washed of the seminal plasma and not capacitated as they do this naturally within the female tract (Suarez and Pacey, 2006).

A previous study investigated the effect of an increasing intracellular cAMP on calcium influx (Torres-Flores et al., 2008). This group used Papaverine, a PDE

inhibitor to increase the cAMP content in non-capacitated sperm. They found that the levels of Ca^{2+} induced by progesterone increased to similar levels to that found in capacitated sperm. With this evidence it may be suggested that the required levels of sAC are maintained even with a lack of HCO_3^- resulting in enough cAMP produced for sperm motility to remain high. However, it is also known that capacitating media contains HCO_3^- which is necessary for sperm capacitation and hyperactivation. Previous studies have carried out investigations on PDE inhibitors and human sperm in media supplemented with bicarbonate (Leclerc et al., 1996) or media without bicarbonate or albumin (Lefievre et al., 2000). A study carried out by Leclerc et al 1996 on cAMP regulation of protein tyrosine phosphorylation on human sperm capacitation and motility, investigated the effect of different media on sperm. They made a comparison between the percent of capacitated sperm when incubated for 4 hours in either Ham's F-10 medium or bicarbonated supplemented Ham's. They found that human sperm capacitation was only stimulated in the presence of bicarbonate when treated with 12-0-tetradecanoyl phorbol 13-acetate (TPA), a protein kinase C agonist known to induce cAMP levels. They also found that in the presence of IBMX, there was an increase in phosphotyrosine content, an effect that was potentiated by bicarbonate. Whether sperm motility increases or decreases depending on the media used, it is crucial that PDE inhibitors maintain their motility enhancing effect if they are to be used clinically. This section investigates the effect of PDE inhibitors on human sperm motility in non-capacitating conditions.

3.1.2 Materials and Methods

Three patient sperm samples were prepared as described in Chapter 2 (2.2.1.2). After centrifugation in HEPES buffered Earle's medium, a dilution was made to the required concentration of approximately $20x10^6$/ml in this non-capacitating medium. Only the 40% fraction was assessed in this case. Samples were incubated with 8-MeOM-IBMX or Rolipram as described in Chapter 2 (2.2.2). Samples were incubated over a period of 4 hours in the HEPES buffered Earle's medium as this is roughly how long it takes for sperm to capacitate in capacitating media. T-test was used in statistical analysis to compare the mean of both control and treated and P value <0.05 was considered significant.

3.1.3 Results

There was a significant increase in the mean percent of motile cells after the addition of 8-MeOM-IBMX at each time point (1, 2, 3 and 4 hours incubation) in HEPES based non-capacitating sperm media (Figure 3.1.1).

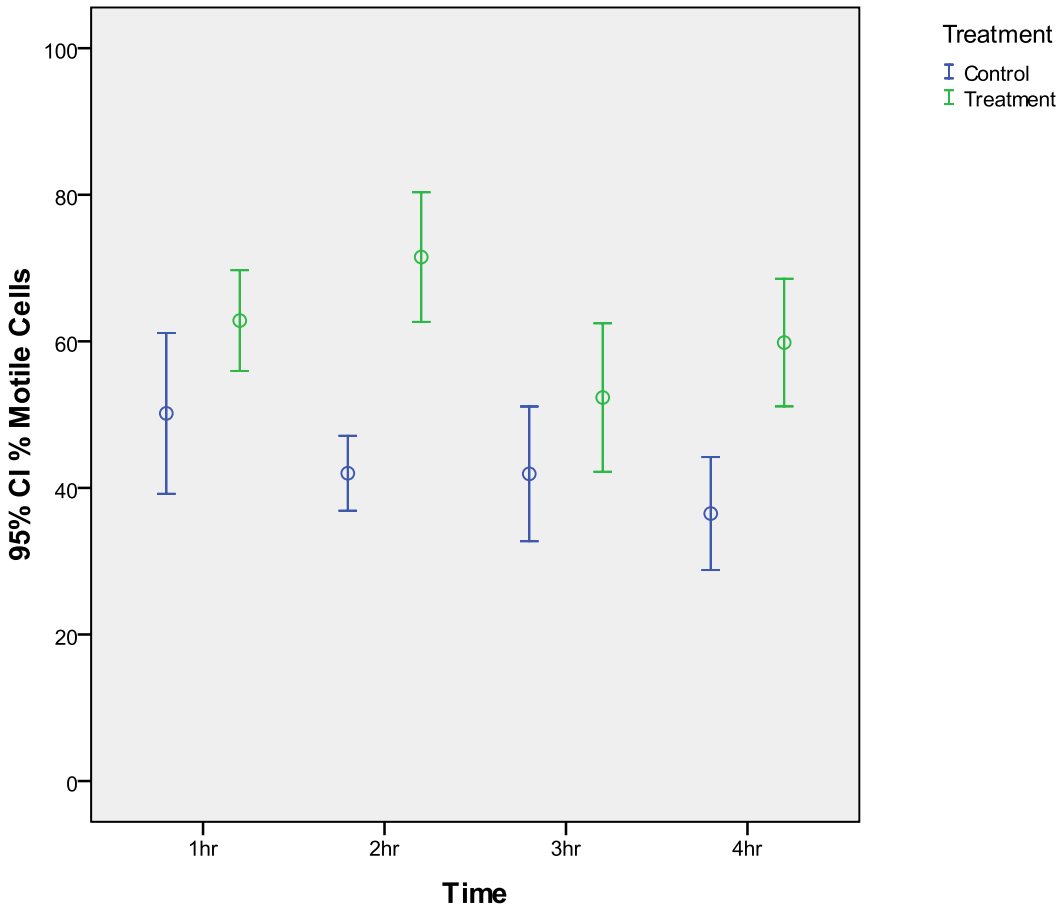

Figure 3.1.1 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different patient sperm samples at 1, 2, 3 and 4 hours incubation with or without the addition of 8-MeOM-IBMX in HEPES based sperm media. $P<0.05$ at each time point and is therefore statistically significant.

There was a significant increase in the mean percent of motile cells after the addition of Rolipram at 1, 2, 3 and 4 hours incubation in HEPES based sperm media (Figure 3.1.2).

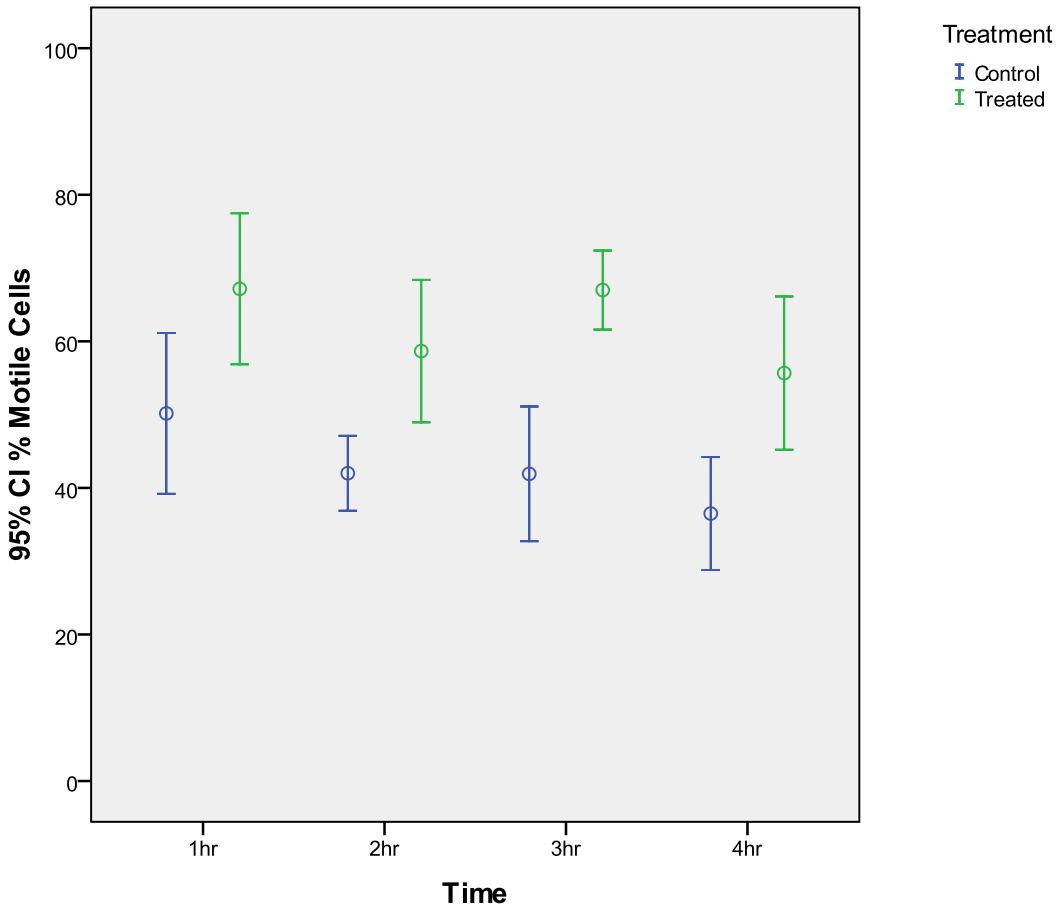

Figure 3.1.2 Each bar represents the mean percent of motile cells in the 40% fraction of 3 different patient sperm samples at 1, 2, 3 and 4 hours incubation with or without the addition of Rolipram in HEPES based sperm media. $P<0.05$ at each time point and is statistically significant.

3.1.4 Discussion

The results shown by figures 3.1.1 and 3.1.2 are similar to the results shown in the previous chapter with regards to the percent of motile cells increasing after the addition of PDE inhibitors. Since this is consistent, the media does not impact upon

the effect of PDE inhibitors on sperm motility of patient samples. Sperm motility is generally lower in non-capacitating media compared to capacitating conditions. Reasons for this may include non-capacitating buffers such as HEPES, do not contain bicarbonate. Bicarbonate is known to change the fluidity of sperm membrane and directly activates sAC (Chen et al., 2000). As it induces these changes to the sperm membrane, the activity of sAC increases the levels of cAMP which then modulates the activity of PKA. Through this signalling pathway, flagellar motility is regulated and capacitation is induced. Notably, PDE inhibitors, which restrict the breakdown of cAMP, are essentially mimicking the effect of bicarbonate. The same can be said for phosphatase inhibitors which prolong PKA-mediated phosphorylation (Gadella and Harrison, 2000). Without bicarbonate in the surrounding sperm media however it is not known as to whether PDE inhibitors would have similar effects on sperm motility as they do in capacitating media. Since bicarbonate is responsible for sperm capacitation and is abundant in the female reproductive tract, it is for this reason sperm are washed in non-capacitating media prior to IUI. This allows the capacitating effects of bicarbonate to take place naturally in the female tract, preventing premature capacitation and possibly acrosome reaction.

The results demonstrate that PDE inhibitors may be used clinically for procedures such as IUI as they maintain their positive effect on sperm motility. The remainder of the study was continued in capacitating conditions. Reasons for this include mimicking the conditions of the female tract to gain a better insight into the effects sperm will have when reaching the oocyte, which will be discussed later in the thesis.

3.2 Effects of varying incubation periods and concentrations of PDE inhibitor on human sperm motility

3.2.1 Introduction

The previous results demonstrate that PDE inhibitors maintain their effect on human sperm motility regardless of the preparation media and their surrounding conditions. The next step was to investigate the effect of the length of sperm and PDE inhibitor incubation time and different PDE inhibitor concentrations.

As sperm swim through the female reproductive tract, it is regulated to ensure that the best quality sperm succeed to the sperm-oocyte interaction stage. It can take hours for sperm to reach the oocyte in humans (Harper et al., 1994), for which sperm must undergo a series of physiological changes and face multiple barriers such as cervical mucus. During this time, sperm must maintain their potential for fertility. Sperm of good motility and normal morphology are more likely to reach the ampulla in the fallopian tubes, (the site of fertilisation), than those of slower progression and/or morphologically abnormal. Enhancing the ability of sperm to swim this long journey could provide possibilities for subfertile men with poor quality samples. These include samples with slow progressing sperm which cannot swim fast and would presumably not last the entire journey to meet the oocyte. Experiments were carried out over an extended incubation period to determine whether PDE inhibitors can maintain their effect on human sperm. If PDE inhibitors were to be used for IUI it would be beneficial to know that their effects on sperm motility can be maintained

until the site of fertilisation. Patient and donor sperm samples were examined in this section since although previous results show that PDE inhibitors do not have a significant effect on donor sperm motility, we do not yet know the effect of these inhibitors over a longer time period. This may be when sperm begin to burn out in the female reproductive tract. It would therefore be useful to know if even good quality sperm from donor samples maintain their progression. The benefit of this would be for couples undergoing IUI.

Sperm samples were also incubated with 8-MeOM-IBMX at either 100µM or 200µM final concentrations to determine whether increasing the concentration of a PDE inhibitor would have more (or less) of an effect on sperm motility. Due to the limited amount of information regarding PDE inhibitors on human sperm motility, it is not known which concentration of PDE inhibitors is best for the sperm. Increasing PDE inhibitor concentration may have a negative or positive effect on the sperm. This is another factor which must be determined if the drugs were to be used clinically.

3.2.2 Materials and Methods

3.2.2.1 Donor sperm preparation

Three different donor sperm samples were prepared as previously described in Chapter 2 (2.2.1.1). As before, 99µl of sperm suspensions were placed in control and two treated labelled eppendorfs. 1µl PDE inhibitor 8-MeOM-IBMX at a final concentration of either 100µM or 200µM was then added to the treated eppendorfs. The mean percent of motile cells from both 40% and 80% fractions, with and without PDE inhibitor was assessed at 1 hour incubation periods up to 8 hours. ANOVA was used in statistical analysis and P value <0.05 was considered significant.

3.2.2.2 Patient sperm preparation

Three different patient sperm samples were prepared as previously described in Chapter 2 (2.2.1.2). As before, 99µl of sperm suspensions were then placed in control and two treated eppendorfs. 1µl PDE inhibitor 8-MeOM-IBMX at a final concentration of either 100µM or 200µM was then added to the treated eppendorfs. The mean percent of motile cells from only the 40% fraction in this case, with and without PDE inhibitor was assessed at 1 hour incubation periods up to 8 hours. ANOVA general linear model was used in statistical analysis and P value <0.05 was considered significant.

Rolipram was also used in this section to determine its effect over a longer incubation period. This was added at a final concentration of 10µM to the 40% fraction of three different patient samples. The mean percent of motile cells was assessed at 1 hour incubation periods up to 8 hours. ANOVA was used in statistical analysis and P value <0.05 was considered significant.

3.2.3 Results

There was no significant difference in the mean percent of motile cells in the 80% fraction of donor sperm using 8-MeOM-IBMX at 0.5, 1 and 2 incubation periods (Figure 3.2.1). However, there was a significant difference during 4, 6 and 8 hour incubation periods between sperm with and without 100µM 8-MeOM-IBMX ($P<0.05$).

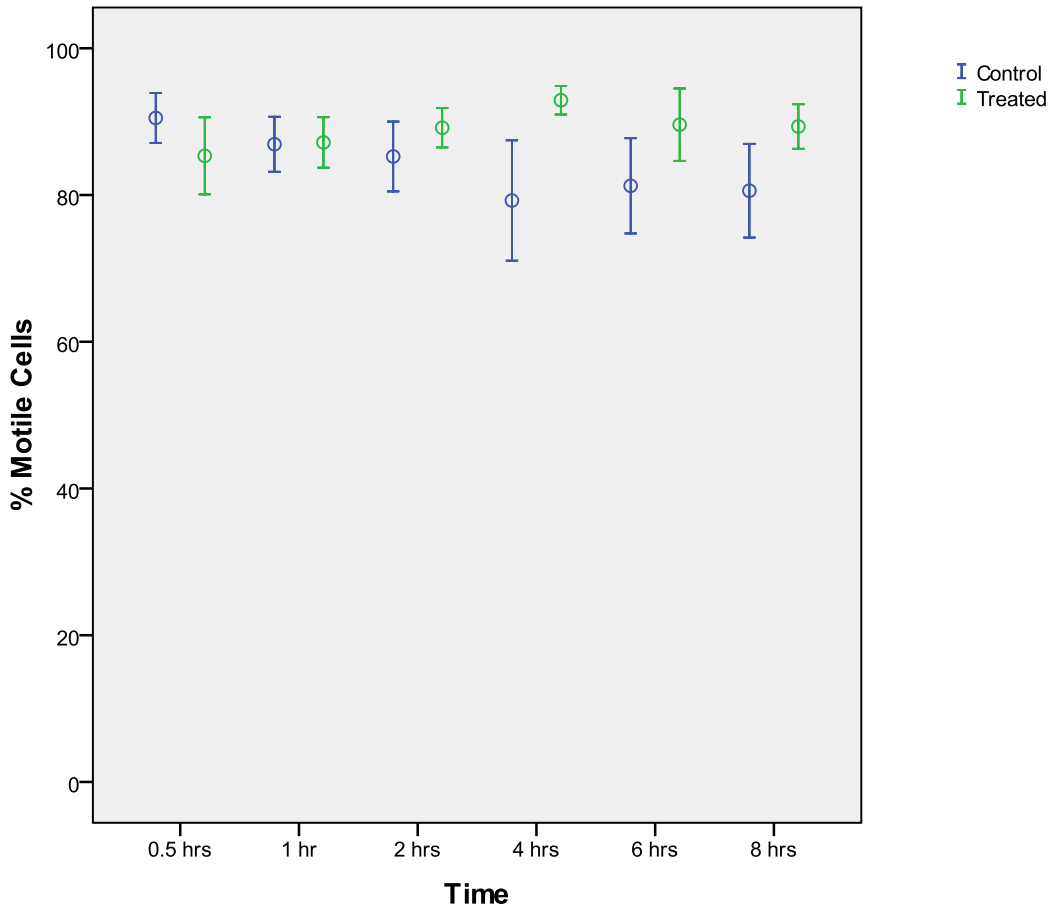

Figure 3.2.1 Each bar represents the mean percent of motile cells from 80% fraction of three different donor samples after incubation with 8-MeOM-IBMX at a final concentration of 100µM between 0.5 and 8 hours.

The same experiment was carried out using a final concentration of 200µM 8-MeOM-IBMX on the 80% fraction of donor sperm (Figure 3.2.2).

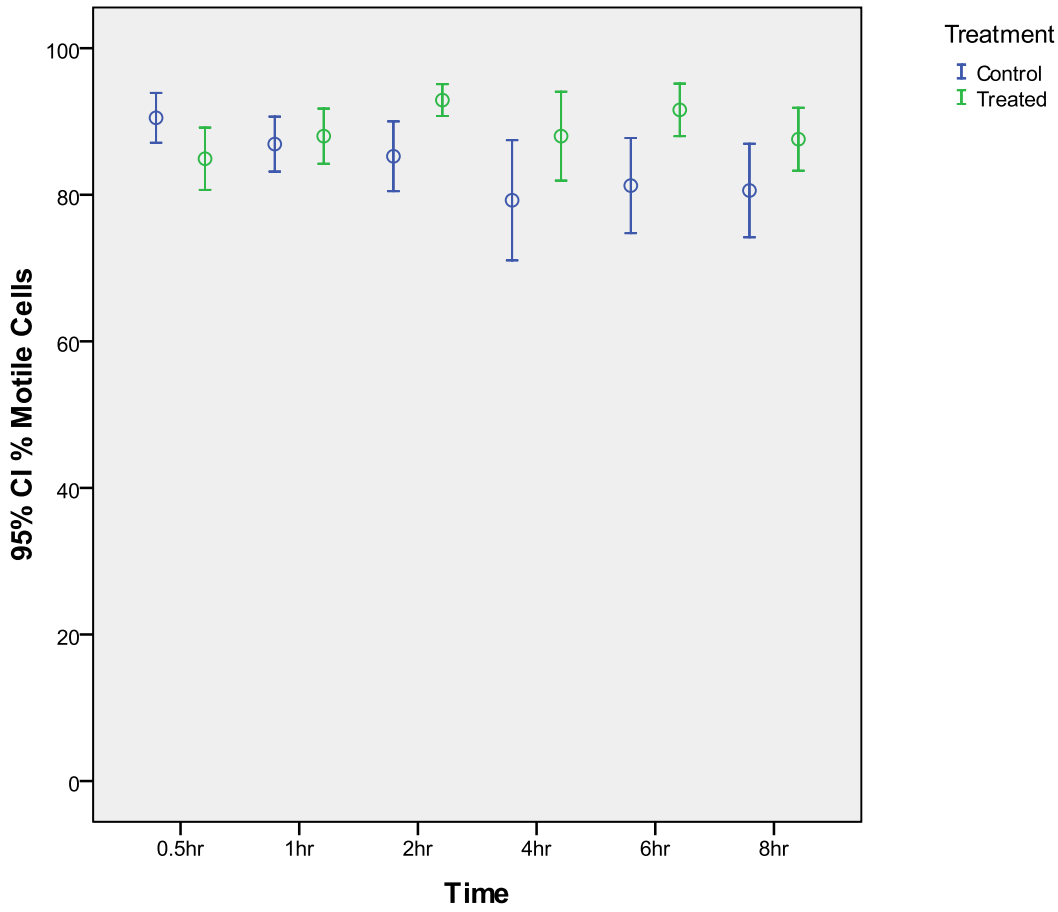

Figure 3.2.2 Each bar represents the mean percent of motile cells from 80% fraction of three different donor samples after incubation with 8-MeOM-IBMX at a final concentration of 200µM between 0.5 and 8 hours.

There was no effect on the mean percent of motile cells at 0.5, 1 and 2 hours of incubation when the 80% fraction of donor sperm were incubated with 8-MeOM-IBMX at a final concentration of 200µM. However, there was a significant difference during 4, 6 and 8 hour incubation periods between sperm with and without 200µM 8-MeOM-IBMX ($P<0.05$).

Increasing the final concentration of 8-MeOM-IBMX to 200µM does not seem to improve sperm motility of the 80% fraction of donor samples more than observed using 8-MeOM-IBMX at a final concentration of 100µM. Following this, the same experiments were carried out on the 40% fraction of donor sperm.

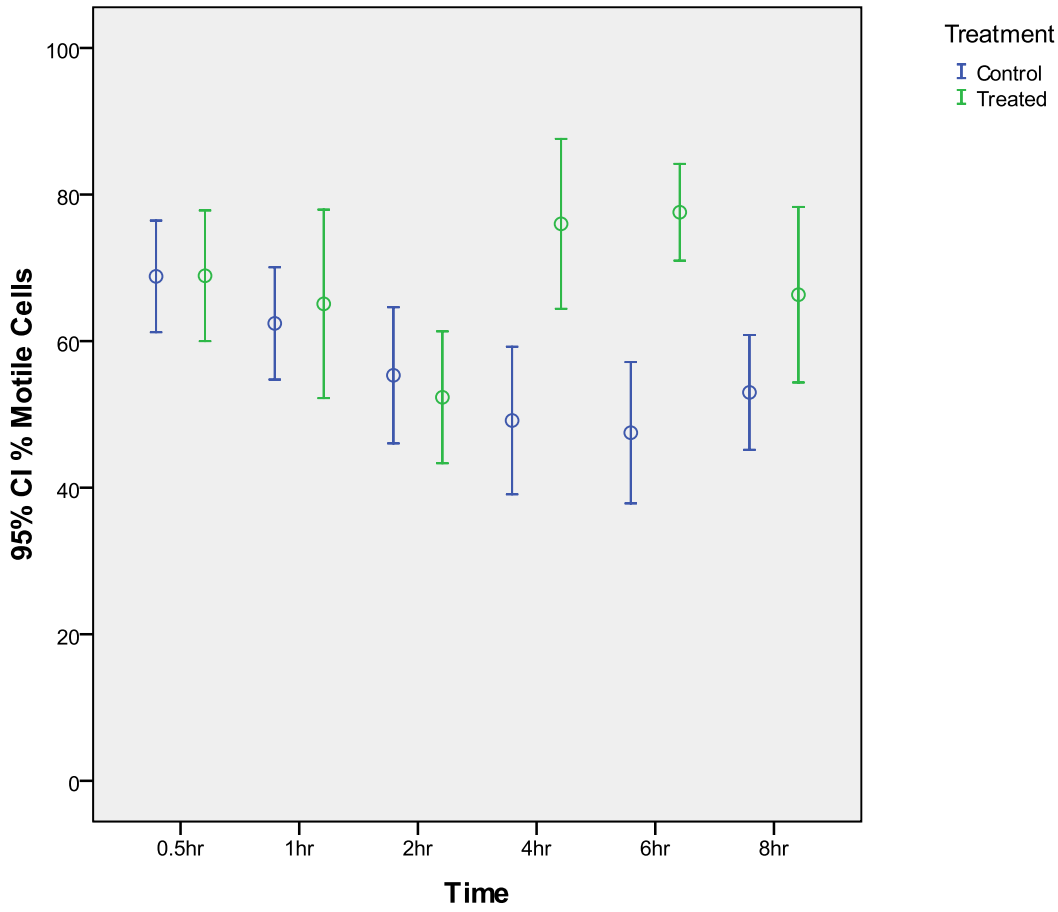

Figure 3.2.3 Each bar represents the mean percent of motile cells from 40% fraction of three different donor samples after incubation with 8-MeOM-IBMX at a final concentration of 100µM between 0.5 and 8 hours.

There was no effect on the mean percent of motile cells at 0.5, 1 and 2 hours of incubation when the 40% fraction of donor sperm were incubated with 8-MeOM-IBMX at a final concentration of 100µM. However, there was a significant difference during 4, 6 and 8 hour incubation periods between sperm with and without 100µM 8-MeOM-IBMX ($P<0.05$) (Figure 3.2.3).

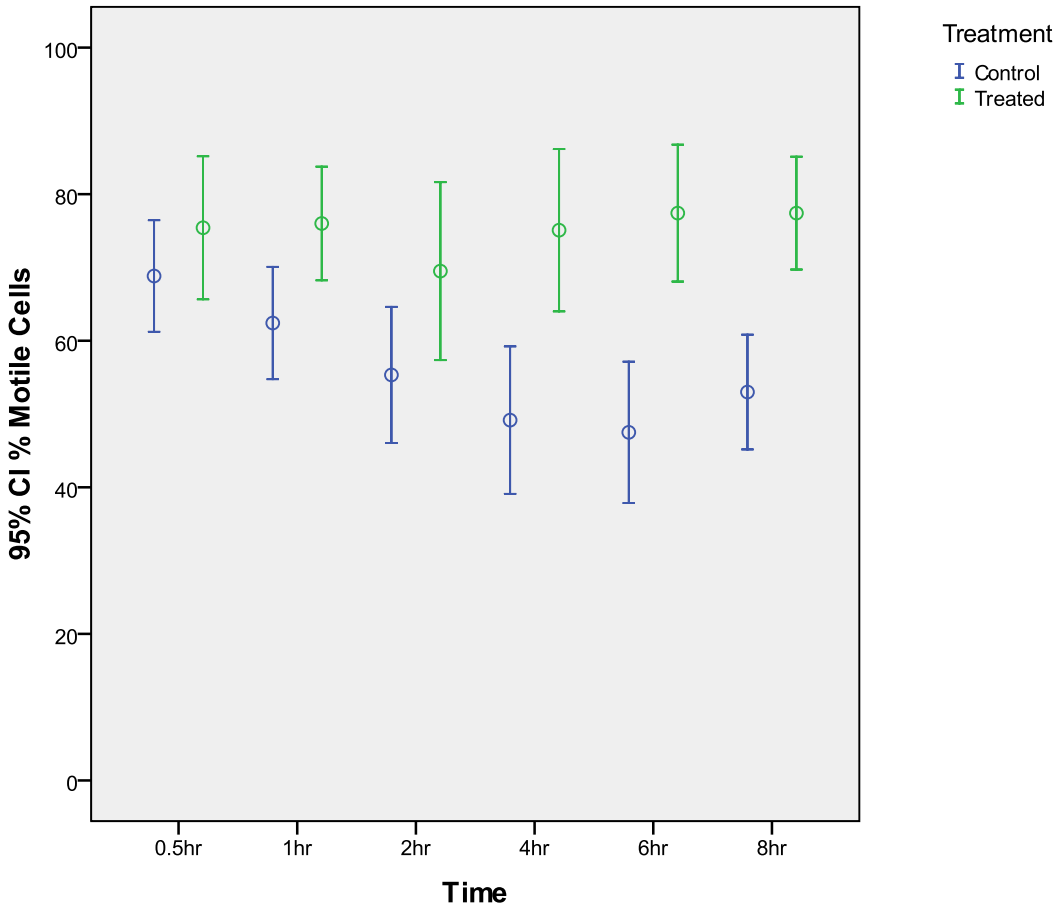

Figure 3.2.4 Each bar represents the mean percent of motile cells from 40% fraction of three different donor samples after incubation with 8-MeOM-IBMX at a final concentration of 200µM between 0.5 and 8 hours.

There was no effect on the mean percent of motile cells at 0.5, 1 and 2 hours of incubation when the 40% fraction of donor sperm were incubated with 8-MeOM-IBMX at a final concentration of 200µM. However, there was a significant difference during 4, 6 and 8 hour incubation periods between sperm with and without 200µM 8-MeOM-IBMX ($P<0.05$) (Figure 3.2.4).

Increasing the final concentration of 8-MeOM-IBMX to 200µM does not seem to improve sperm motility of the 40% fraction of donor samples more than observed using 8-MeOM-IBMX at a final concentration of 100µM. Following this, the mean percent of motile cells in the 40% fraction of patient samples was also investigated through an 8 hour incubation period after the addition of 8-MeOM-IBMX at a final concentration of 100µM (Figure 3.2.5).

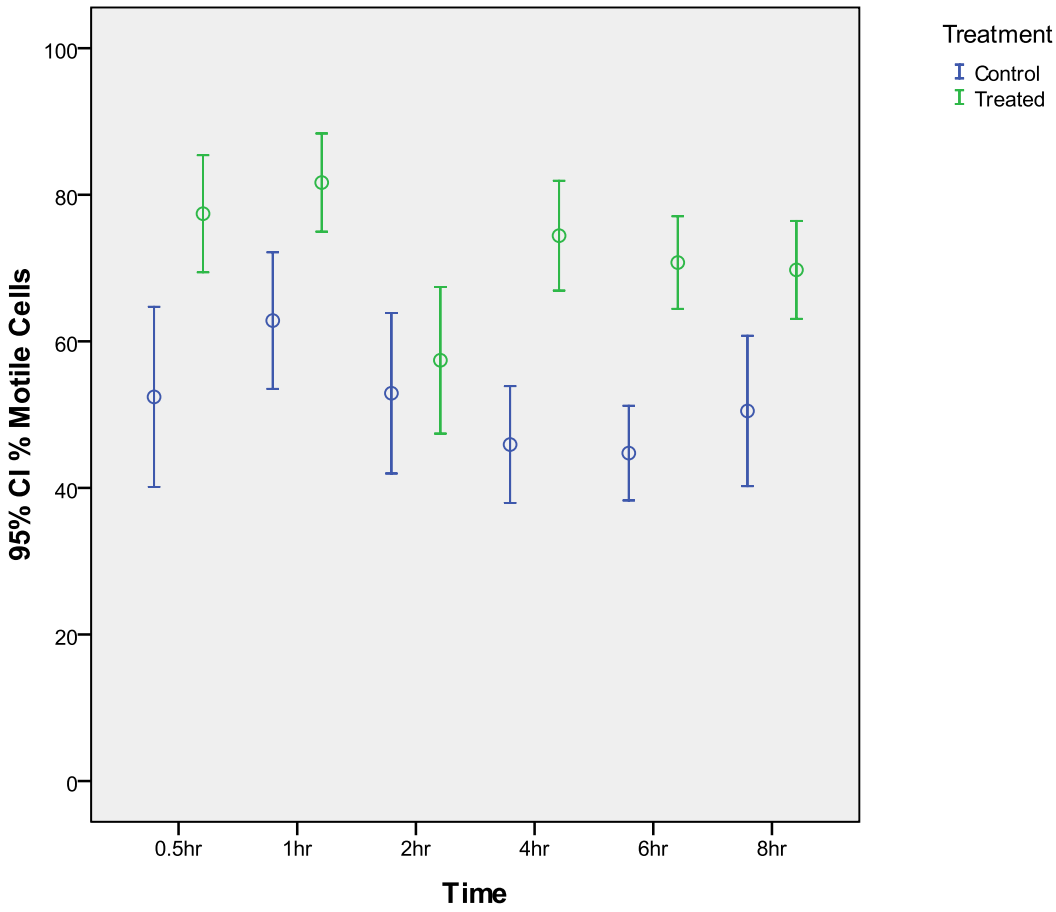

Figure 3.2.5 Each bar represents the mean percent of motile cells from 40% fraction of three different patient sampels after incubation with 8-MeOM-IBMX at a final concentration of 100µM between 0.5 and 8 hours.

There was a significant increase ($P<0.05$) in the mean percent of motile cells in the 40% fraction of patient samples after the addition of 100µM 8-MeOM-IBMX which remained constant between 0.5 and 8 hours of incubation apart from the 2 hour incubation period. The next step was to investigate the effect of increasing the final concentration of 8-MeOM-IBMX to 200µM on the mean percent of motile sperm in the 40% fraction of patient sperm samples.

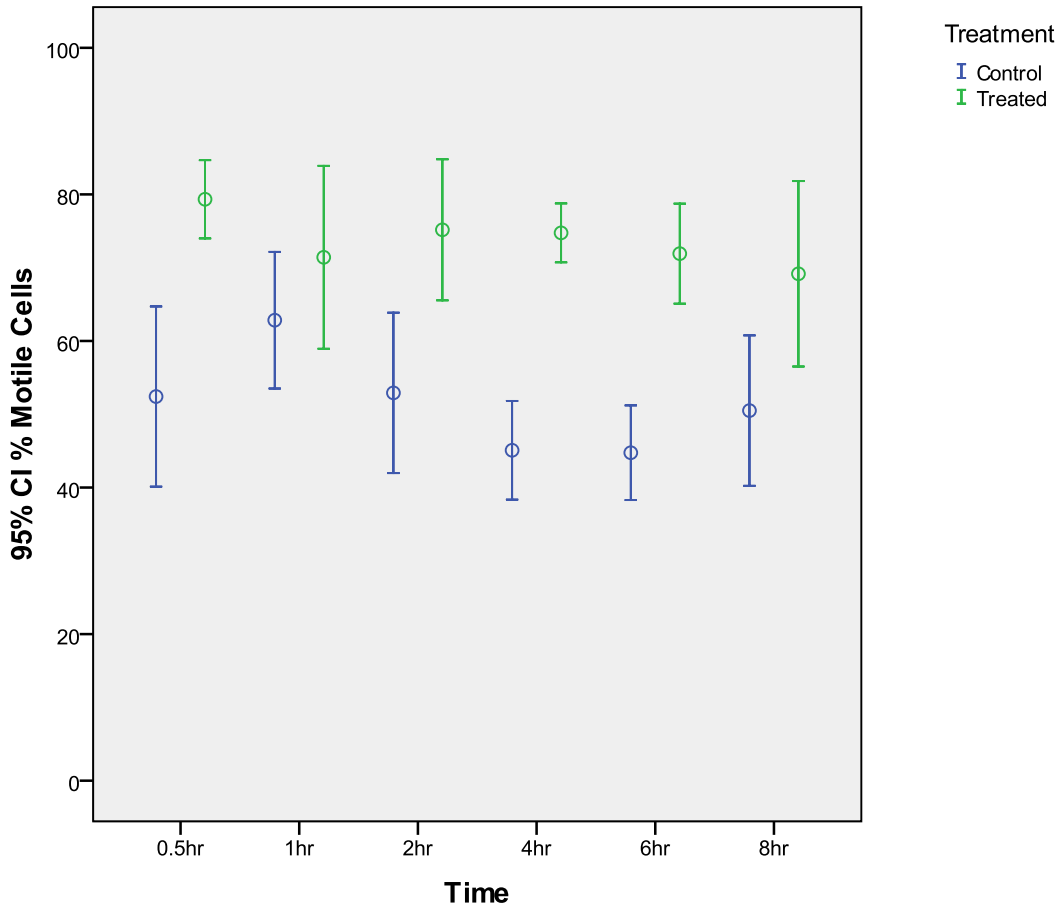

Figure 3.2.6 Each bar represents the mean percent of motile cells from 40% fraction of three different patient sampels after incubation with 8-MeOM-IBMX at a final concentration of 200µM between 0.5 and 8 hours.

There was a significant increase ($P<0.05$) in the mean percent of motile cells in the 40% fraction of patient samples after the addition of 200µM 8-MeOM-IBMX which remained constant between 0.5 and 8 hours of incubation apart from the 1 hour incubation period (Figure 3.2.6).

Increasing the final concentration of 8-MeOM-IBMX to 200µM does not seem to improve sperm motility of the 40% fraction of patient samples more than observed using 8-MeOM-IBMX at a final concentration of 100µM. Following this, an 8 hour incubation period was carried out using PDE-4 inhibitor Rolipram at a final concentration of 10 µM on the 40% fraction of patient samples to investigate the effect on the mean percent of motile cells (Figure 3.2.7).

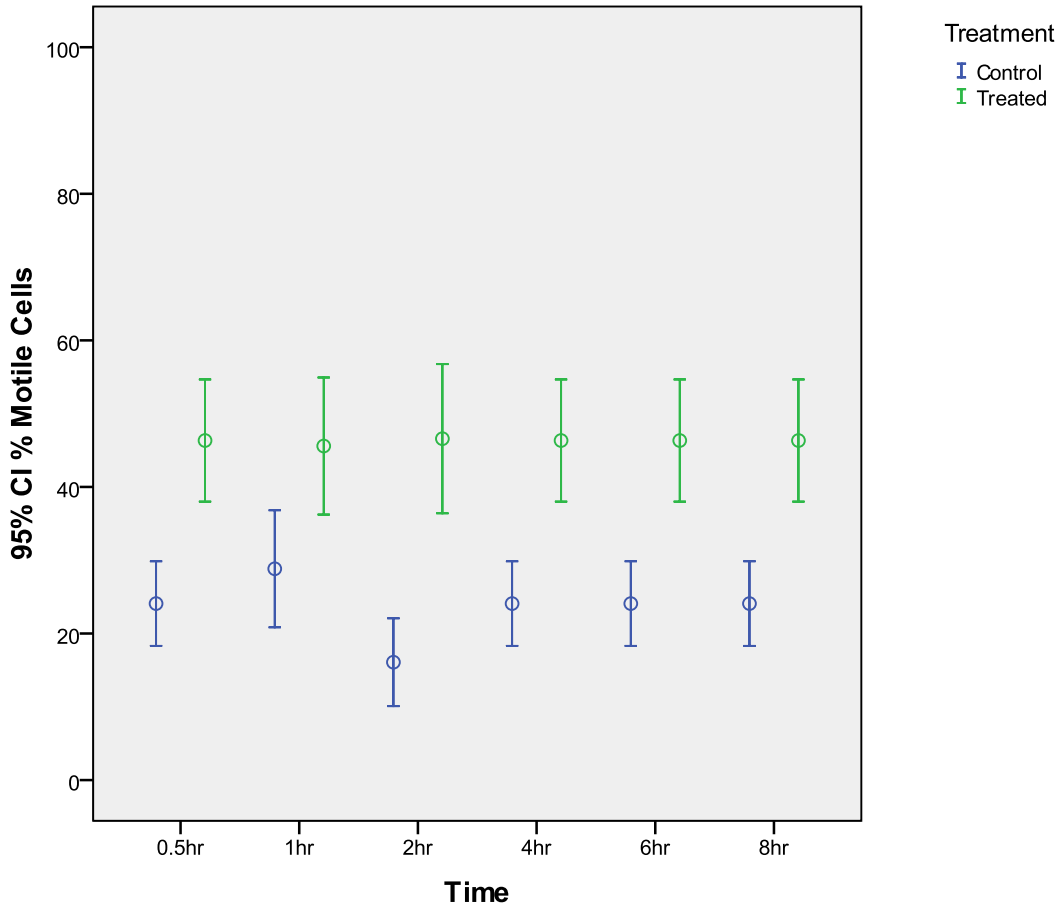

Figure 3.2.7 Each bar represents the mean percent of motile cells from 40% fraction of three different patient sampels after incubation with Rolipram at a final concentration of 10µM between 0.5 and 8 hours.

There was a significant increase ($P<0.05$) in the mean percent of motile cells in the 40% fraction of patient samples after the addition of 10µM Rolipram which remained constant between 0.5 and 8 hours of incubation (Figure 3.2.7).

3.2.4 Discussion

For sperm to reach the oocyte it is essential that they can survive the journey through the female reproductive tract. For good quality sperm this may be a case of the motile count of a samples or maintaining progressive motility for a number of hours to ensure sperm-oocyte interaction takes place. For poor quality sperm such as samples from sub-fertile men, the number of motile cells in a sample may be the determining factor for getting to the site of fertilisation.

The above results suggest as before that 8-MeOM-IBMX does not have an effect on the percent of motile cells in donor sperm samples (Chapter 2 2.3.1), and that 8-MeOM-IBMX does have an effect on the percent of motile cells in patient sperm samples (Chapter 2 2.3.2) over a couple of hours. In addition to this, the results show that PDE inhibitor 8-MeOM-IBMX has an effect on patient sperm over 8 hours of incubation not only on patient sperm but also donor samples at longer time periods. This may be beneficial even for perfectly normal sperm samples in cases such as IUI since it can take hours for sperm to reach the oocyte and may prevent sperm burning out. Donor sperm for IUI procedures is commonly used as a frozen-thawed sample which is known to have reduced semen quality (Lim, et al 2010) due to the freeze-

thaw process. The average success rates for donor insemination are relatively low with women under the age of 35 having a success rate of 15.3% in the UK in 2008 (HFEA, 2010). If PDE inhibitors can maintain their effects on sperm motility for longer time periods this may increase the success rate of IUI altogether.

Increasing the final concentration of 8-MeOM-IBMX to 200µM had no further effect when compared to the effect of using 100µM (as used in Chapter 2) on either donor or patient sperm over any time period. This suggests that 100µM may be the optimal concentration for increasing human sperm motility. However, such limited information is available on the effect of PDEs on human sperm that investigating various concentrations may be a consideration of further research. It is therefore unknown as to what the optimal concentration of any PDE inhibitor is on human sperm with regards to improving motility and function. It is also unknown as to whether any damage may be caused by increasing PDE inhibitor concentration on the sperm cell. The results however suggest that although increasing the concentration of 8-MeOM-IBMX does not improve sperm motility, it does not appear to have any negative effect on the percent of motile sperm within a sample. This may be a small amount of information but may be valuable in the investigation of PDE inhibitors on human sperm.

3.3 The effect of combining PDE inhibitors on human sperm motility

3.3.1 Introduction

Previous studies carried out using PDE inhibitors on sperm have investigated the inhibitors individually. In other words, concentrating on the effects of one PDE inhibitor at a time on sperm. Reasons for this may include the fact that generally little is known about the effect of PDE inhibitors on human sperm and so a more thorough investigation is carried out using one inhibitor. Although studies have shown PDE inhibitors have a strong positive effect on human sperm motility, further information is yet to be determined such as varying concentrations or extended incubation periods as discussed previously. Since this study suggests that individual PDE inhibitors increase the percent of motile cells in patient samples, it is important to determine whether this effect can be further advanced (or if it negatively effects sperm) simply by incubating patient sperm with more than one inhibitor. No study to date has examined the effect of combinding more than one PDE inhibitor on human sperm motility. In this section, the 40% fraction of patient sperm samples were incubated with both 8-MeOM-IBMX and Rolipram (as used in previous sections) in order to determine the effect of combining PDE inhibitors on the percent of motile cells of patient sperm samples.

3.3.2 Materials and Methods

Three different patient sperm samples were prepared as described previously (Chapter 2 2.2.1.2). Only the 40% fraction was examined in this section. The sperm suspension was assessed at 0.5, 1 and 2 hour incubation periods with the addition of both 8-MeOM-IBMX and Rolipram at final concentrations of 100µM and 10µM respectively. ANOVA general linear model was used in statistical analysis and P value <0.05 was considered significant.

3.3.3 Results

The effect of adding 8-MeOM-IBMX or Rolipram individually to the 40% fraction of patient sperm samples significantly ($P<0.05$) increased the mean percent of motile sperm at the 0.5, 1 and 2 hour incubation periods (Figure 3.3.1) as demonstrated in previous sections. The combined effect of both 8-MeOM-IBMX and Rolipram had a significant ($P<0.05$) effect by increasing the mean percent of motile cells of the 40% fraction of patient sperm at 0.5, 1 and 2 hour incubation periods.

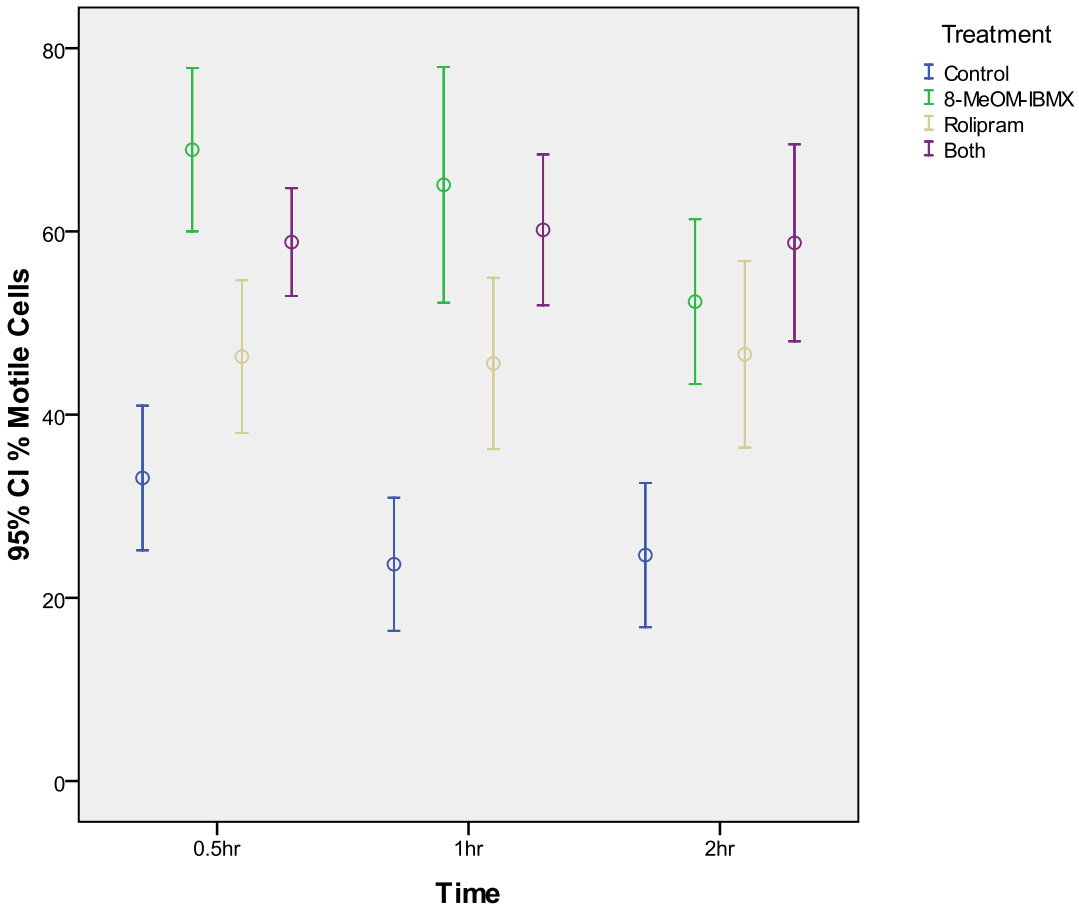

Figure 3.3.1. Graph demonstrating the mean percent of motile cells when the 40% fraction of three different patient sperm samples are incubated with either 8-MeOM-IBMX, Rolipram and both at 0.5, 1 and 2 hour incubation periods.

3.3.4 Discussion

The results show consistency with previous sections in that using 8-MeOM-IBMX or Rolipram individually has a significant positive effect on the motility of patient sperm samples. The results of combining both 8-MeOM-IBMX and Rolipram also significantly increases the percent of motile sperm in patient samples. This increase

was greater than the effect of Rolipram alone. This suggests that the use of two inhibitors rather than one has a positive effect on sperm motility, and more importantly this does not seem to have a negative effect on human sperm motility. However, the effect of both inhibitors on the percent of motile cells combined is less than that of 8-MeOM-IBMX. This suggests the opposite in that the use of both inhibitors may be an overload for the sperm cell in terms of a possible build up of the levels of cAMP, essentially causing the sperm to burn out. If so, this may prevent the use of combining PDE inhibitors clinically (or at least certain PDE inhibitors) since what appears to be a negative effect on patient sperm. More investigation on the effect of combining PDE inhibitors on sperm motiltiy is required using different PDE inhibitors on a larger population of sub-fertile patients. As before, these results may be skewed by the small numbers examined and the variation between samples. However, in conclusion to these results it would appear as though a combination of PDE inhibitors does improve sperm motility, although not to the extent of one inhibitor alone.

3.4 The effect of washing sperm after PDE inhibitor incubation

3.4.1 Introduction

Clinical sperm preparation consists of ‘washing’ the sperm in gamete buffer to remove any remaining PureSperm® silica particles after gradient centrifugation. Washing sperm also gets rid of any dead or slow moving sperm along with these

addition chemicals. This is done to remove what may impair fertilisation. If PDE inhibitors leave a residue in the media surrounding the sperm, it may be crucial that PDE inhibitors are added before a wash if PDE inhibitors were to be used in a clinical setting. This would wash patient sperm from their surrounding media to eliminate the chance of PDE inhibitors being transmitted into the uterus in such cases as preparation for IUI. Preparation for IVF insemination would also require the PDE inhibitor surrounding media to be washed as this media would be inseminated along with patient sperm into the insemination dish containing oocytes. Previous studies investigating the effects of PDE inhibition on human sperm motility have not washed the sperm suspensions after adding PDE inhibitors (Lefievre et al., 2002). Reasons for this may include the fact that generally little is known about the effect of PDE inhibitors on sperm and we are not yet at the stage of investigating alternatives in sperm preparation, such as the effect of adding a further wash step. However it is important when investigating the effect of PDE inhibitors on human sperm to gain as much knowledge as possible and determine whether the positive effects of PDE inhibitors on patient sperm motility are maintained.

3.4.2 Materials and Methods

Three different patient sperm samples were collected and prepared as described previously (Chapter 2 2.2.1.2). After the addition of PDE inhibitor 8-MeOM-IBMX or Rolipram at final concentrations of 100µM and 10µM respectively, the sperm suspensions were incubated for 1 hour at 37°C with 6% CO_2. These suspensions

were then placed in 5ml pre-equilibrated STF medium and density gradient centrifugated again for 10 minutes at 500g. Following this extra wash step, the media was taken off as far as the pellet and the pellet was suspended in pre-equilibrated STF to a final concentration of approximately $20x10^6$/ml for assessment using CASA. T-test was used in statistical analysis and P value <0.05 was considered significant.

3.4.3 Results

The results demonstrate that there is no significant difference (P>0.05) between the mean percent of motile cells from the 40% fraction of patient sperm following 1 hour incubation of either 8-MeOM-IBMX or Rolipram following an additional wash step (Figure 3.4.1). The percent of motile cells for samples which had an additional wash step were high and similar to that of samples without an extra wash.

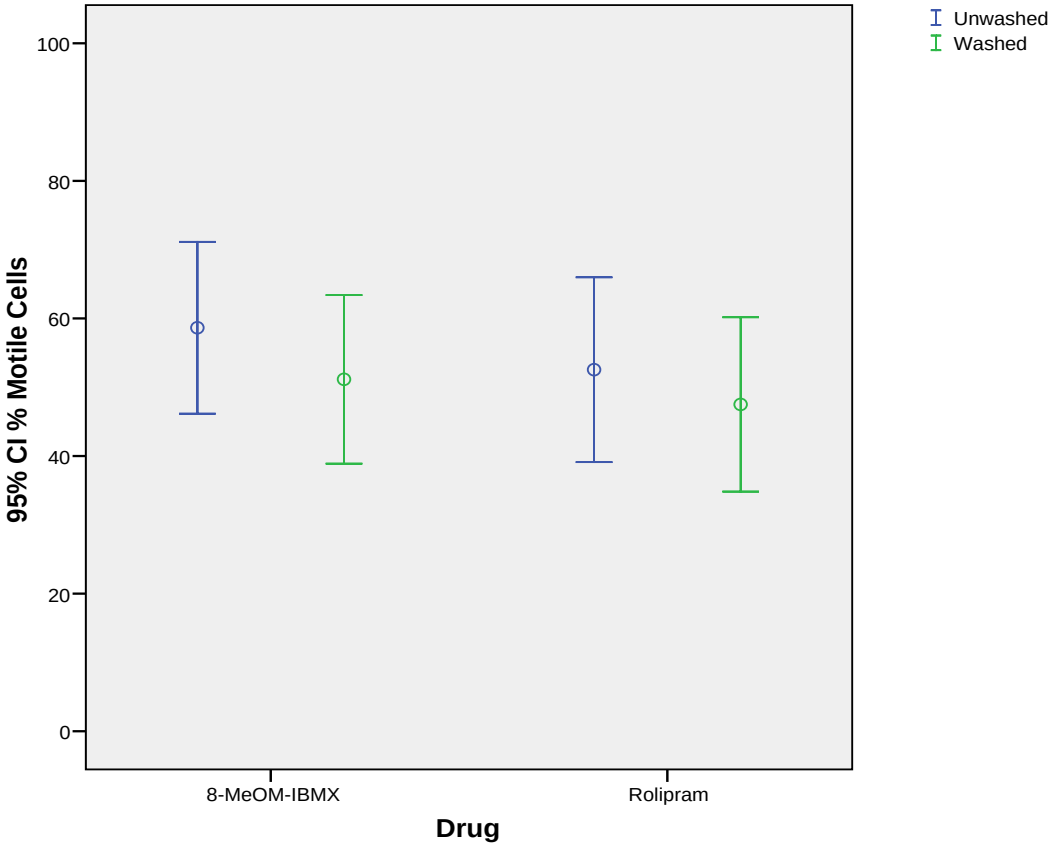

Figure 3.4.1 Graph comparing the mean percent of motile cells between unwashed and washed sperm from the 40% fractions of three different patient semen samples after incubation with 8-MeOM-IBMX or Rolipram at final concentrations of 100µM and 10µM respectively for 1 hour.

3.4.4 Discussion

The resuts suggest that introducing an additional wash stage in the preparation of patient sperm samples when using PDE inhibitors would have no additional effect (positive or negative) on patient sperm. The benefit of this is knowing that if it is a requirement that PDE inhibitors must be washed from the surrounding media, they

maintain their positive effect on patient sperm motility. This would also be convenient during patient sperm preparation as PDE inhibitors may be added during the sperm preparation procedure which could save time in an embryology laboratory prior to insemination. Since the results demonstrate that PDE inhibitors maintain their effects after the removal of any unwanted PDE inhibitor from the surrounding media after 1 hour incubation, this almost ensures the PDE inhibitor remains within the cell.

One of the most important aspects of this result is that washing sperm after PDE inhibitor incubation has no negative effect on sperm motility. Experiments carried out previously in this study have mainly demonstrated a positive relationship between PDE inhibitors and sperm motility. However, due to the limited amount of information on PDE inhibitors in human sperm, little is known about the effect they may have on any functional and physiological damage on sperm. It would appear thus far that PDE inhibitors do not have a negative effect on sperm however this has only been concluded through studies regarding the maintainence of motility (Fisch et al., 1998). Sperm damage may include induced change to the acrosomal status of a sperm head or induced sperm DNA damage. This may not only affect the ability of sperm to bind to the oocyte, but also fertilisation results. If adding PDE inhibitors to increase the fertilisation potential of sperm proves to have a negative effect on sperm in any way, this will prohibit the use of the inhibitors clinically. The next section explores the effects of PDE inhibitors on sperm function and potentially the fertilising ability of human sperm.

Chapter 4: Sperm physiology and PDE inhibitors

4.1 Acrosome Reaction

4.1.1 Introduction

The acrosome of mammalian sperm is a membranous exocytotic sac containing many kinds of hydrolytic enzymes (Harper et al., 2008). It plays a crucial role during sperm-oocyte interaction. Freshly ejaculated sperm must acquire the ability to fertilise an oocyte which is done by a series of changes known as capacitation. Capacitation involves changes such as modifications to plasma membrane lipid composition and the acquisition of hyperactivated motility (Lishko et al., 2011). The acrosome reaction can only happen in capacitated sperm, enabling them to eventually fuse with the oocyte. Mitchell et al, 2007 demonstrated that capacitation is associated with an increase in sperm-zona pellucida binding. This study added zona-pellucida from human oocytes to sperm from capacitating or non-capacitating conditions and counted the number of sperm bound using phase contrast and fluorescence microscopy. They showed significantly more sperm were able to bind to zona-pellucida after allowing them to capacitate. This demonstrates the importance of capacitation during this step in fertilisation. During the acrosome reaction, acrosomal contents such as hydrolytic enzymes and acrosomal antigens are gradually realised in intermediate stages as sperm develop the potential to fuse to the surface of the oocyte. As the outer acrosome membrane of the sperm fuses with the oocyte the

acrosomal contents along with the inner acrosomal membrane are exposed to the extracellular medium (Bhandari et al., 2010).

There is debate as to when the acrosome reaction actually takes place during sperm-oocyte interaction and the consequences this may have on the ability of sperm to fertilise. For instance, it has been suggested that human sperm that have undergone premature acrosome reaction cannot fertilise (Liu and Baker, 1990). However, it is also suggested that acrosome reacted sperm within the vicinity of the oocyte are capable of fertilisation (Morales et al., 1989). Other studies have also suggested that in various species such as mice, sperm can only initiate binding to the zona pellucida if acrosome intact (Saling and Storey, 1979; Florman and Storey, 1982; Bleil and Wassarman, 1983). However, recent studies have shown that most fertilising sperm undergo the acrosome reaction before reaching the zona-pellucida (Jin et al., 2011). Using fluorescence microscopy, this study evaluated the acrosome reaction during sperm-oocyte interaction in mice sperm and oocytes, providing further debate on the stage of sperm acrosome reaction and the need for a better understanding of sperm physiology during fertilisation. However, it is important to remember that the physiology of sperm-oocyte interaction may be species specific and what applies to mice may not be the same for human gametes.

The acrosome reaction plays a key role in the sperm-oocyte interaction (Cross et al., 1988). Recent studies have explored various factors as to what promotes this physiological change. It has been suggested that human zona pellucida can induce acrosomal exocytosis (Ganguly et al., 2010). The human zona pellucida is an extracellular glycoprotein coat which is composed of four main glycoproteins. It acts

as an agonist for acrosomal exocytosis of the sperm head and plays an important role in the protection of the oocyte in maintaining cytoplasmic contents and preventing polyspermy. The precise function of each of the four glycoproteins (ZP1, ZP2, ZP3 and ZP4) in human zona pellucida has been a topic of great interest. It has previously been demonstrated that in human oocytes, ZP3 and ZP4 binds to capacitated acrosome intact sperm and induces the acrosome reaction (Chiu et al., 2008). More recently ZP1 has also been shown to bind to capacitated acrosome intact sperm, inducing the acrosome reaction (Ganguly et al., 2010). It has previously been shown however that ZP2 binds to the acrosome and does not induce the acrosomal exocytosis in capacitated human sperm (Chakravarty et al., 2008). Acrosomal exocytosis in human sperm may be a combined effect of all three zona pellucida glycoproteins binding to sperm and inducting the acrosome reaction. Further studies are required to determine the exact role of the zona pellucida on the acrosome status of human sperm and when exactly the acrosome reaction should take place in order to achieve fertilisation.

It has previously been suggested that premature acrosome reaction may counteract the effects of the ability of sperm to fertilise an oocyte (Tesarik et al., 1992). Promotion of the acrosome reaction may therefore not be beneficial in improving fertilisation rates. Whilst it is important that sperm undergo this physiological change, it is also important that PDE inhibitors do not promote the acrosome reaction due to the chance of it being detrimental to sperm-oocyte interaction. There has previously been correlation between PDE inhibitors and the acrosome reaction. Fisch et al 1998 demonstrated a significant stimulating effect on sperm acrosome with the

use of PDE-1 inhibitor 8-MeOM-IBMX. This would make sense since PDE-1 is predominantly found in the sperm head (Lefievre et al., 2002). In contrast, PDE-4 inhibitor Rolipram provoked a significant improvement in sperm motility yet did not affect the acrosome. However, whilst these results look clinically positive, studies have demonstrated a negative effect on the use of PDE inhibitors on sperm acrosome and in turn sperm function. Glenn et al 2007 demonstrated the PDE-5 inhibitor Sildenafil induced a significant increase in the percent of acrosome reacted sperm. In contrast, Lefièvre et al 2000 reported that Sildenafil increases sperm motility, capacitation, protein tyrosine phosphorylation but not the acrosome reaction. Due to constant debate, the acrosome reaction must be evaluated to determine whether PDE inhibitors have any effect on the acrosomal status of human sperm cells. Even though this subject is still controversial, if it is clear that PDE inhibitors induce the acrosome reaction they would not be used clinically due to these unwanted effects. PDE inhibitors 8-MeOM-IBMX and Rolipram were used in this section as these are the inhibitors which have shown so far in this study to have the greatest effect on sperm motility. If these inhibitors provoke the acrosome reaction they would not be used clinically due to the risk of premature acrosome reaction negatively influencing fertilisation success.

4.1.2 Materials and Methods

The 80% fraction of three different donor samples was used in this section. They were collected and prepared as previously described (Chapter 2 2.2.1.1). Following

sperm preparation the samples were suspended at a concentration of approximately $10x10^6$M/ml in 0.5ml pre-equilibrated STF. The sperm suspensions were incubated at 37°C, 6% CO_2 for 3 hours to allow capacitation. Three 1.5ml eppendorfs were prepared of control, positive control and treated. After incubation, 0.5ml suspended sample was added to each eppendorf. For the control, 0.5µl DMSO was added giving 0.1% DMSO (only if PDE inhibitor used was diluted in DMSO). For the positive control, 2.5µl of Ca^{2+} ionophore A23187 was added at a final concentration of 10µM (Fisch., et al 1998). For the 'treated' sample, 5µl of either 8-MeOM-IBMX or Rolipram was added (PDE inhibitors diluted to required final concentration beforehand). The 3 eppendorfs were then incubated at 37°C at 6% CO_2 for 60 minutes. 2.5µl of propidium iodide was then added to each eppendorf at a final concentration of 12µM and left for 15 minutes at 37°C. The samples were then washed by centrifugation for 10 minutes at 500g and the supernatant discarded. The pellet was suspended in 200µl STF before transferring to slides. 20µl of the sperm suspension was transferred from each eppendorf to poly-L-lysine coated slides and left for 30 minutes to air dry. To make the sperm cells permeable they were immersed in 100% methanol for 1 minute at room temperature and dried immediately on a heated stage to rapidly evaporate the methanol. 25µl fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) was then added to each slide at a concentration of 50µg/ml (Fisch., et al 1998). Slides were incubated for 1 hour in a moisture chamber at 37°C before being washed in a gentle stream of water for 2-3 minutes. Slides were then mounted and left to air dry in the dark at room temperature for at least 30 minutes before being evaluated. Evaluation was carried out by counting 200 cells per slide in duplicate. Cells were then viewed under

a fluorescence microscope (40x objective) and counted as acrosome intact live cells, acrosome reacted live cells, acrosome intact dead cells and acrosome reacted dead cells (Cross, 1986). Data on the sperm acrosome reaction test is presented as a mean percent from the proportion of living sperm. T-test was used in statistical analysis and P value <0.05 was considered significant.

4.1.3 Results

The results shown are taken from the live cell count only. The comparison between the percent of cells acrosome intact and acrosome reacted is clear between the negative and positive control (Figure 4.1). The average percentage of acrosome reacted sperm in the negative control was 11.75% (± 1.3% SEM) whereas the positive control treated with Ca^{2+} ionophore A23187 showed 45% (± 7.9% SEM) (Table 1). The results are statistically significant (P<0.05). Rolipram at a final concentration of 10µM did not have a significant effect on the acrosome reaction of sperm. When compared with the negative control the difference in the percent of acrosome reacted sperm was 0.5% (± 1.3% SEM) (P>0.05) thus showing no clear stimulating effects. 8-MeOM-IBMX at 100µM however showed a small but significant result. When compared with the negative control, the difference in the percent of acrosome reacted sperm was 3.08% (± 0.58% SEM) (P=0.042).

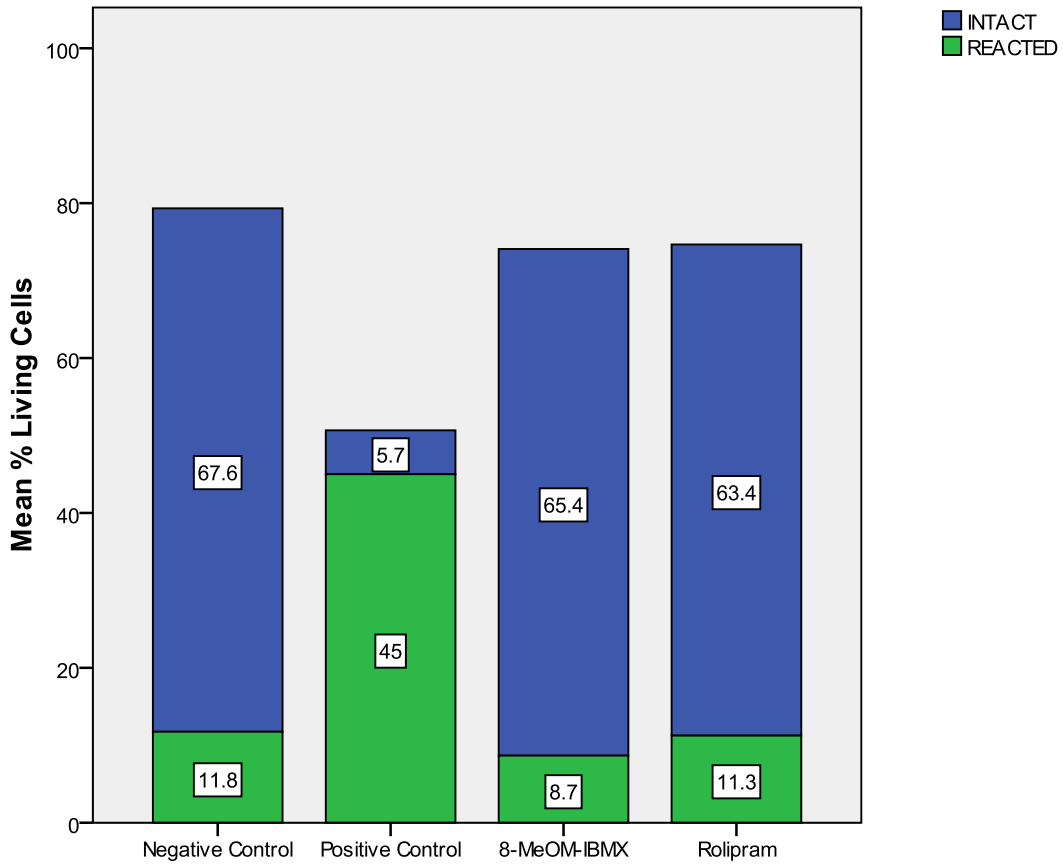

Figure 4.1 Graph representing the mean percent of live cells acrosome intact and reacted from the 80% fraction of three different donor sperm samples after 1 hour incubation with either 8-MeOM-IBMX or Rolipram at final concentration of 100µM and 10µM respectively.

Compound	PDE Inhibited	% Reacted cells (mean ± SEM)	P value Control vs. treated
Negative control (0.1% DMSO)	-	11.75 ± 1.3	-
Positive control (10µM A23187)	-	45 ± 7.9	-
8-MeOM-IBMX (100µM)	PDE-1	8.67 ± 0.72	P=0.042
Rolipram (10µM)	PDE-4	11.25 ± 2.61	P=0.58

Table 4.1 Table demonstrates the effect of 8-MeOM-IBMX and Rolipram on the acrosomal status of human sperm.

4.1.4 Discussion

This study was carried out in order to determine whether or not PDE inhibitors have an effect on the acrosomal status of human sperm. The results gained suggested that PDE inhibitors 8-MeOM-IBMX and Rolipram do not induce the acrosome reaction in human sperm compared to what occurs naturally. It has long been thought that sperm must acrosome react within the vicinity of the oocyte in order to go on and penetrate the oocyte. The site at which the acrosome reaction takes place is still an on-going debate and the stability of sperm after they have acrosome reacted is unknown. However, it is known that sperm must undergo the acrosome reaction in order to penetrate and fertilise the oocyte.

Previous studies have observed an increase in acrosome reacted sperm when incubated with PDE inhibitors suggesting the inhibitors are involved in promoting this reaction. Reasons for this may be due to the use of non-selective PDE inhibitors such at PTX. PDE inhibitors such as PTX have been used to stimulate sperm motility (Yovich et al., 1990). Tasdemir et al 1993 used PTX to evaluate any possible effects on sperm acrosome reaction. They found that PTX did induce the acrosome reaction significantly in patient samples. However, they also found a positive relationship between PTX treated sperm and fertilisation rates. Other reasons for previous correlation between PDE inhibitors and acrosome reaction are the concentration of non-selective PDE inhibitors used. PDE inhibitor concentrations previously used to achieve an increase in human sperm motility have been shown to be accompanied by an increase in the acrosome reaction. Fisch et al used PDE-4 type inhibitor Rolipram at 10µM which showed no effect on the number of acrosome reacted sperm compared to the non selective IBMX used at 100µM which seemed to significantly induce the acrosome reaction.

It is important that the acrosome reaction is not initiated until the sperm are in the vicinity of the oocyte (Tesarik, 1989). This may not be crucial during IVF since sperm are placed in the vicinity of the oocyte. However, for IUI this may be what determines fertilisation outcome as sperm have far to travel before reaching the oocyte. Even though this subject is still controversial, it is important that PDE inhibitors do not have any negative effects on sperm function. The purpose of using PDE inhibitors is to improve the quality of sperm from infertile patients, in turn increasing the fertilisation potential of these couples. It is therefore important that

sperm are both morphologically and physiologically sound to function well in order to fertilise the oocyte.

Some sperm infertility features however are not as apparent as the presence or absence of an acrosome. Sperm have underlying physiological issues, some of which include DNA damage. Detection of DNA damage is beyond that of a routine semen analysis but the ability to do so allows identification of one of the most important fertilisation determinants. It is suggested that sperm cell DNA damage may be caused or inherited. It is therefore important that no damage is caused to sperm DNA with the use of PDE inhibitors.

4.2 Tunel Assay

4.2.1 Introduction

An important determinant of male infertility is DNA damage (Bungum et al., 2007). The sperm from some infertile men have structural and functional defects (Liu and Baker 1994). These defects include DNA fragmentation which frequently affects sperm by either hindering or preventing fertilisation. While sperm appear motile and morphologically normal, they may be genetically abnormal. The mechanisms of DNA damage include apoptosis, oxidative stress and defective chromatin packing. In some cases these abnormalities may be caused by environmental factors such as exposure to chemical and/or physical agents (Friedler, 1996). This can alter the

genome at the chromosomal or DNA level leading to abnormalities in the nucleoprotein content or DNA strand breaks. It has also been suggested that sperm DNA damage is inherited since fragmentation of DNA is associated with structural chromosomal abnormalities (Perrin et al., 2011). Sperm with DNA damage do not have the capacity for fertilisation. It has been demonstrated that with couples undergoing IUI, none of the semen samples with >12% sperm DNA fragmentation resulted in pregnancy (Duran et al., 2002). If DNA damaged sperm were to be injected into the oocyte, this may also have serious consequences on the offspring (Ahmadi and Ng, 1999).

The TUNEL assay was first developed in the 1990s (Muratori et al., 2010) and first applied to human sperm in 1993 (Gorczyca et al., 1993). Problems with this technique include the heterogeneity of sperm DNA fragmentation. For example freshly ejaculated semen samples from groups of similar infertile subjects can vary from 2.4% (n=29) to 39.8% (n=66) DNA fragmentation (Younglai et al., 2001; Domínguez-Fandos et al., 2007). Due to unreliable results and lack of standardisation, male fertility status is difficult to predict using this technique. However, conflicting results suggest that the diagnostic and prognostic value of sperm DNA fragmentation has not been concluded and therefore with an established procedure, results may be precise (Muratori et al., 2010). The TUNEL assay is in fact used today for DNA fragmentation evaluation of sperm. Piasecka et al 2007 assessed the genomic integrity of asthenozoospermia patients in relation to their fertilisation potential. They discovered that the proportion of TUNEL-positive cells (those with DNA fragmentation) was significantly higher in the sperm of those patients with

lower motility (n=40). They also found a correlation between TUNEL-positive cells and other factors such as immature sperm, mid-piece abnormalities and ultra structural chromatin. Other studies such as Chiamchanya et al 2010 used the TUNEL assay to determine the percent of DNA damage after various density gradient centrifugations, revealing PureSperm® as yielding the best motility and the lowest percentage of protamine deficiency. These abnormalities can therefore be evaluated using the TUNEL assay and may help select sperm which are actually healthy and eliminate those with underlying abnormalities.

It is therefore important to determine the nature of PDE inhibitors and if they have a negative effect on the DNA component of sperm. If there is a toxic effect and DNA damage is induced with the addition of PDE inhibitors then it is important that these drugs are not used since they will not only affect fertilisation rates but will most likely be harmful to the patient and any resulting offspring.

4.2.2 Materials and Methods

The 80% fraction of three different donor samples was used in this section. They were collected and prepared as previously described (Chapter 2 2.2.1.1). After sperm preparation, samples were suspended in 0.5ml HEPES based media at a sperm concentration of approximately $1x10^7$M/ml. 8-MeOM-IBMX or Rolipram was added at final concentrations of 100µM or 10µM respectively and left at room temperature for 1 hour. Poly-L-lysine slides were prepared of negative control, positive control,

non-treated and treated. 50µl of the samples were placed onto the slides and smeared with a cover slip. The slides were left to air dry for 3 hours at room temperature. Once dry, a circle was drawn around the sample using a wax pen. The sample was then fixed in 100µl 4% Paraformaldehyde for 15 minutes. The slides were then washed in PBS wash bath for 15 minutes. Solution of proteinase K and Tris buffer (1 in 100 dilution) was made up and 50µl placed on slide. This was left for 5 minutes at room temperature and then washed in PBS. 50µl DNase1 was then place on positive control whilst 100µl DNase1 solution placed on all other slides. All slides were left for 20 minutes at room temperature and then washed in PBS. 100µl of a solution containing TdT and dH_20 (1 in 5 dilution) was added to each slide and left at 20 minutes room temperature. 60µl of a solution containing Reaction Mix and TdT Enzyme were added to each slide besides the negative control, to which 60µl Reaction Mix alone was added. Slides were covered in parafilm and placed in moisture chambers for 1-1.5 hours at 37°C. Parafilm was then taken off and the slides were washed in PBS for 1 minute. Slides were mounted using mounting media with cover slips. Mounted samples were stored at 4°C in the dark. T-test was used in statistical analysis and P value <0.05 was considered significant.

4.2.3 Results

Results from the negative and positive controls are not recorded here. The negative control which should have an amount of DNA damage natural to the sample since it did not have the TdT enzyme added to it had 0.67 ± 0.08% DNA damaged cells.

There was a significant difference compared to the positive control carried out by the DNase1 enzyme which showed 87.04 ± 3.4% DNA damaged cells. The difference between the negative and positive controls is 86.38 ± 3.32% and is therefore statistically significant ($P<0.05$).

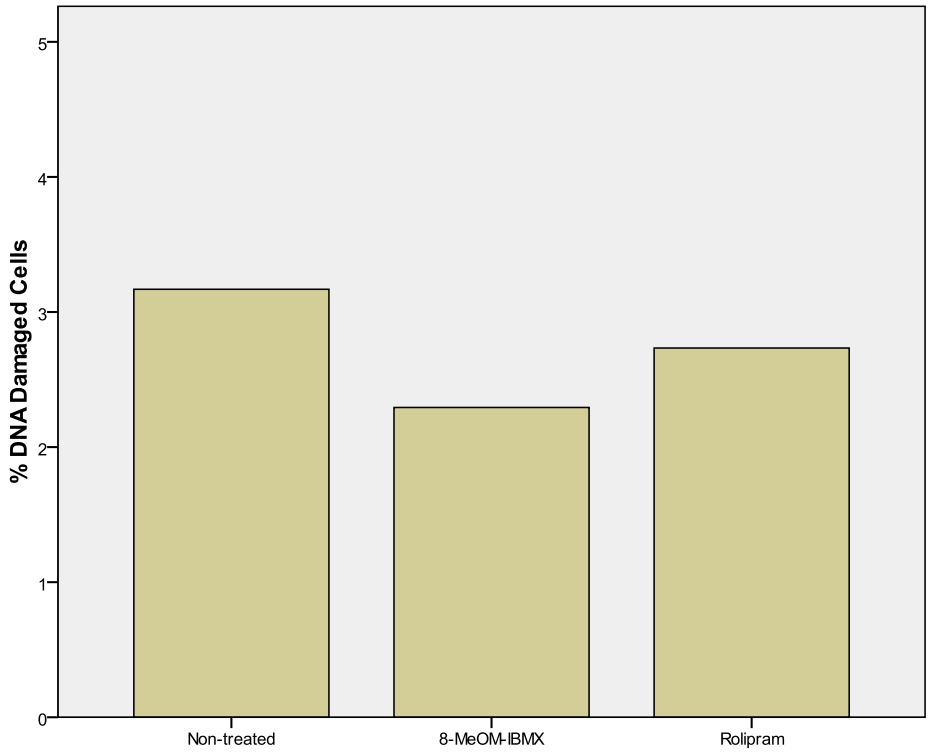

Figure 4.2 Graph represents the mean percent of cells with DNA damage from the 80% fraction of three different donor sperm samples. A comparison between non treated and PDE inhibitor treated samples.

There was no significant difference (P>0.05) in the mean percent of DNA damaged cells between the control and either 8-MeOM-IBMX or Rolipram treated sperm of the 80% fraction from donor samples (figure 4.2).

4.2.4 Discussion

Human sperm DNA damage is a prognostic factor of male infertility and is increasingly being recognised as a tool in predicting embryo development. Conventional parameters of semen analysis do not take sufficient information into account for a complete evaluation of a couples reproductive potential. DNA damage testing helps to detect defects in sperm quality. Today this has a particular importance due to the growing concern regarding the transmission of genetic diseases. The process of natural selection is bypassed with the use of ICSI allowing DNA damaged sperm to fertilise oocytes. Factors that impact upon the DNA integrity of a sperm cell may have consequences for any resulting embryo, blastocyst or even child. These results clearly demonstrate that PDE inhibitors do not have a negative effect on sperm DNA. Causes of DNA damage in sperm include abnormal chromatin packaging, reactive oxygen species and apoptosis (Shamsi et al., 2008). It is therefore clinically beneficial that PDE inhibitors do not fall into this category. However, these experiments were carried out on relatively low samples numbers and require a much larger population to gain a more reliable result. The TUNEL assay itself is also not a reliable test due to previous reported unreliable results and requires standardisation. It would also be useful to carry out further investigation on patient

sperm samples since sub-fertile men appear to be the target population for the use of PDEs.

Chapter 5: Further investigation on the effect of PDE inhibitors on human sperm motility

5.1 Introduction

It is surprising that even though studies have been carried out on human sperm , including sub-fertile patient samples, still nothing has been done to fully investigate the potential of PDE inhibitors. In this chapter, three PDE type specific inhibitors have been chosen to investigate the effect of PDE inhibitors in more depth than what has previously been carried out. One PDE inhibitor Milrinone, which is selective to PDE-3 already located in human sperm, is reported to have no effect on sperm motility, (Lefievre et al., 2002) another Papaverine which has an effect on human sperm motility yet has not been located in human sperm and lastly BRL which has never been studied in human or animal sperm. There is limited information available on these three inhibitors which are investigated in this section. It is due to ths limited knowledge that these three PDE inhibitors was chosen. These findings may produce novel results, whether they have a positive effect on sperm motility or not.

PDE-3A (an isoform of PDE-3) has been located in the postacrosomal segment of human sperm head (Lefievre et al., 2002). This study investigated the cAMP content of human sperm using Milrinone. The level of cAMP increased with the use of Milrinone at 50μM however this did not affect sperm functions such as capacitation, protein phosphorylation and motility when compared to IBMX as a positive control. Nevertheless, both localization and inhibition confirmed the presence of PDE-3 in

human sperm and also confirmed Milrinone as a PDE-3 specific inhibitor. In contrast, Papaverine, an inhibitor of PDE-10 has been shown to have a significant effect on human sperm function yet has never been localized. Torres-Flores et al 2008 examined the effect of exposing non capacitated human sperm to Papaverine. They found that calcium levels increased to levels close to that of capacitated sperm. They also found a greater effect on sperm calcium levels using Papaverine than PTX, a widely used PDE inhibitor for sperm motility enhancement. Since it has long been known that calcium is an important regulator of sperm motility, (Tash and Means, 1983) the increase caused by Papaverine suggests that this PDE inhibitor may also regulate sperm motility.

Little has been done to investigate the effects of PDE inhibitors in human sperm particularly with the knowledge that there is a correlation between PDE inhibition and sperm motility enhancement. These inhibitors have been chosen on that basis for further investigation along with BRL, an inhibitor of PDE-7 which has never before been studied in sperm.

5.2 Materials and Methods

The 40% fraction of three patient sperm samples was used in this study. Samples were collected and prepared as described previously (Chapter 2 2.2.1.2). PDE inhibitors Milrinone, BRL and Papaverine were used at final concentrations of 50µM, 100µM and 100µM respectively. Sperm suspensions were incubated for 0.5,

1 and 2 hours at 37°C with 6% CO_2. The mean percent of sperm motility and progressively motile cells were assessed using CASA. ANOVA was used in statistical analysis and P value <0.05 was considered significant.

5.3 Results

The results demonstrate a significant (P<0.05) increase in the mean percent of motile cells after incubation with each of the PDE inhibitors at 0.5, 1 and 2 hour incubation periods. Samples incubated with Milrinone showed an increase particulary at 1 hour with the control having an average percent motility of 42.2% ± 10.6 whilst the treated was 66.1% ± 8.7 (P<0.05) (Figure 5.1). Similarly BRL had a significant effect on the 40% fraction of patient sperm. The control had an average motility of 42.2% ± 10.6 compared to the treated which had 71.3% ± 10.0 (P<0.05) at 1 hour incubation period (Figure 5.3). Papaverine also demonstrated a significant result when added to the 40% fraction of patient samples. The average motility of the control was 42.2% ± 10.6 compared to the treated samples which was 65.8% ± 12.3 (P<0.05) at 1 hour incubation period (Figure 5.5). The average percent of progressively motile cells was significantly higher (P<0.05) throughout 0.5, 1 and 2 hours for Milrinone, BRL and Papaverine (Figures 5.2, 5.4 and 5.6).

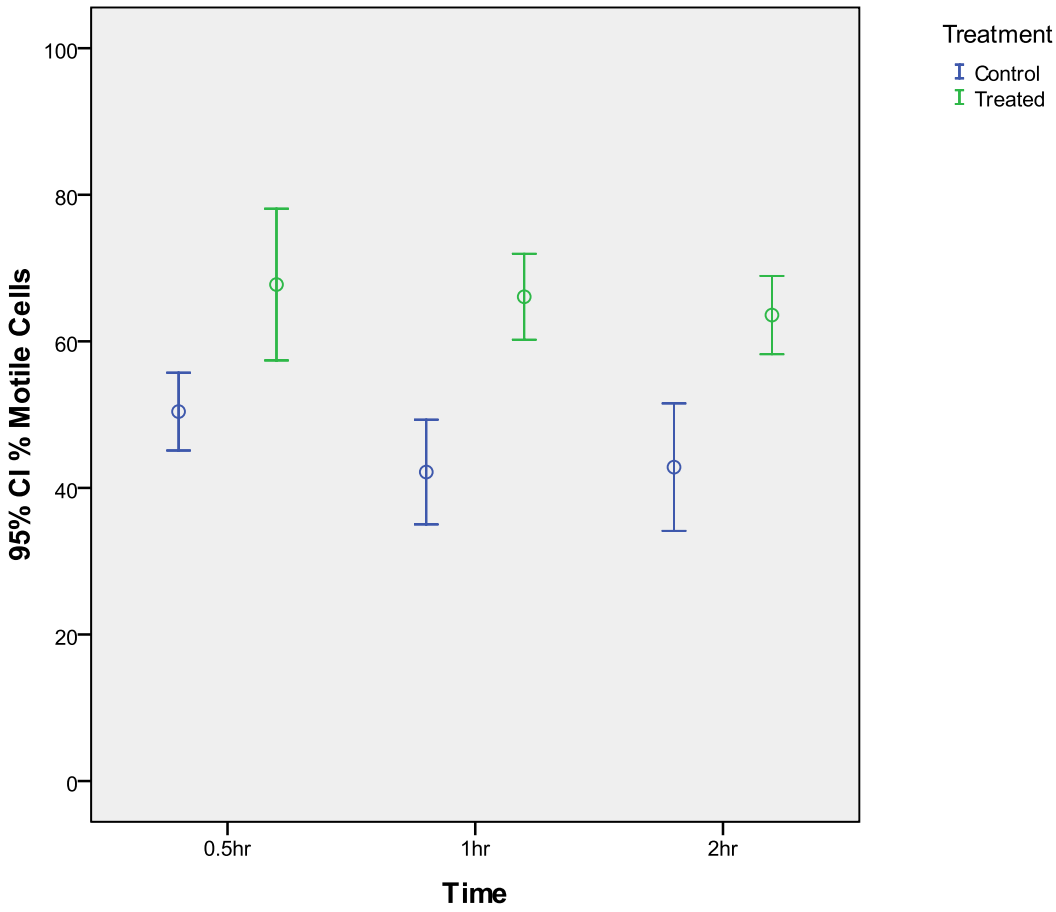

Figure 5.1 Each bar represents the mean percent of motile cells with or without the addition of Milrinone on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

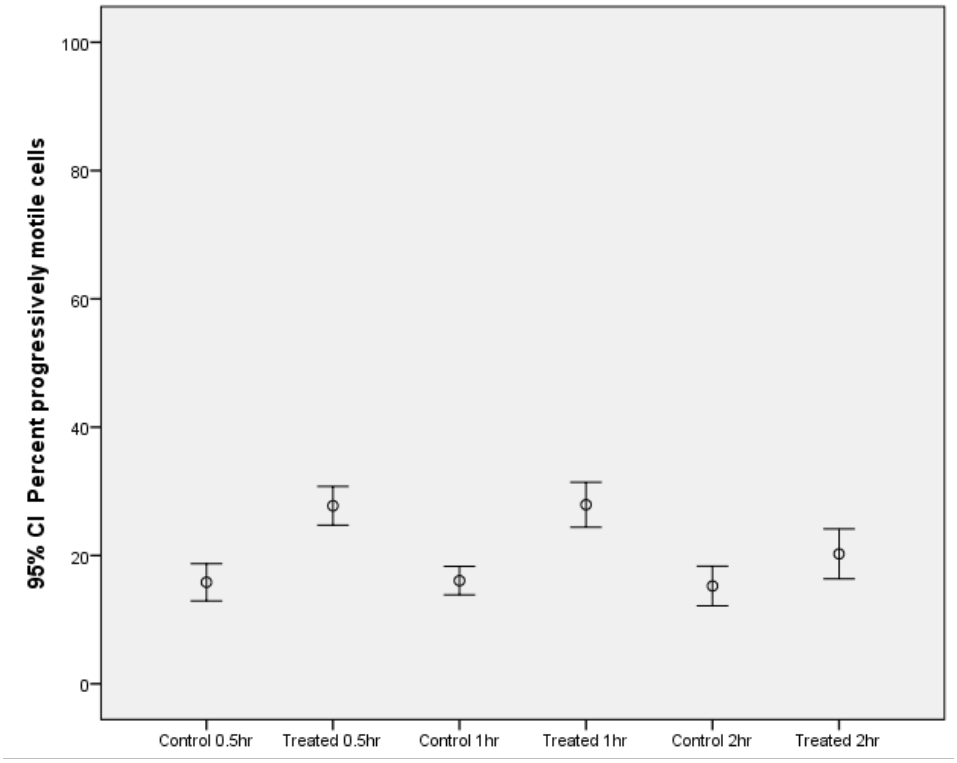

Figure 5.2 Each bar represents the mean percent of progressively motile cells with or without the addition of Milrinone on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

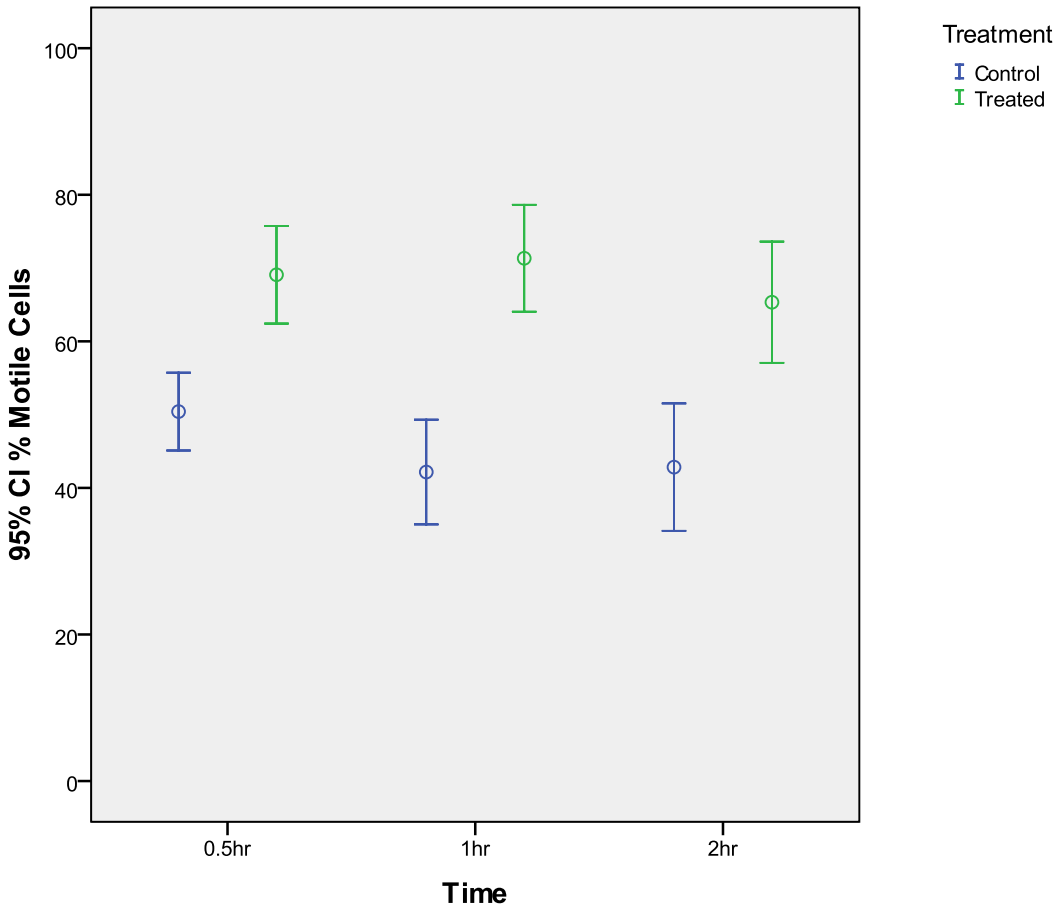

Figure 5.3 Each bar represents the mean percent of motile cells with or without the addition of BRL on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

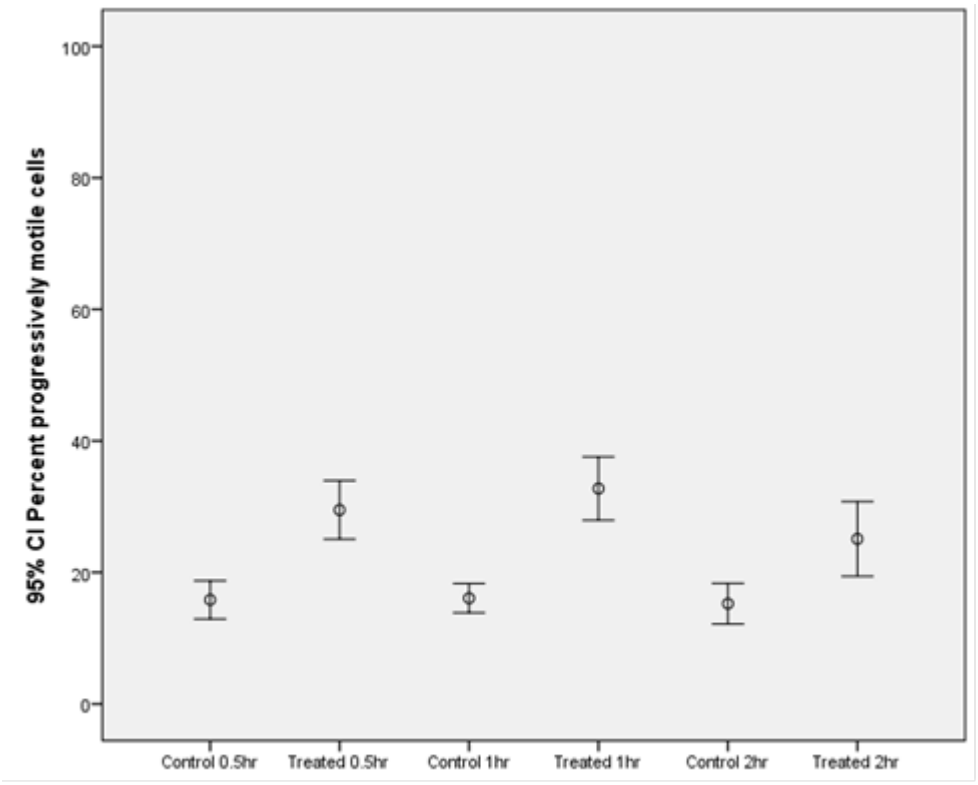

Figure 5.4 Each bar represents the mean percent of progressively motile cells with or without the addition of BRL on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

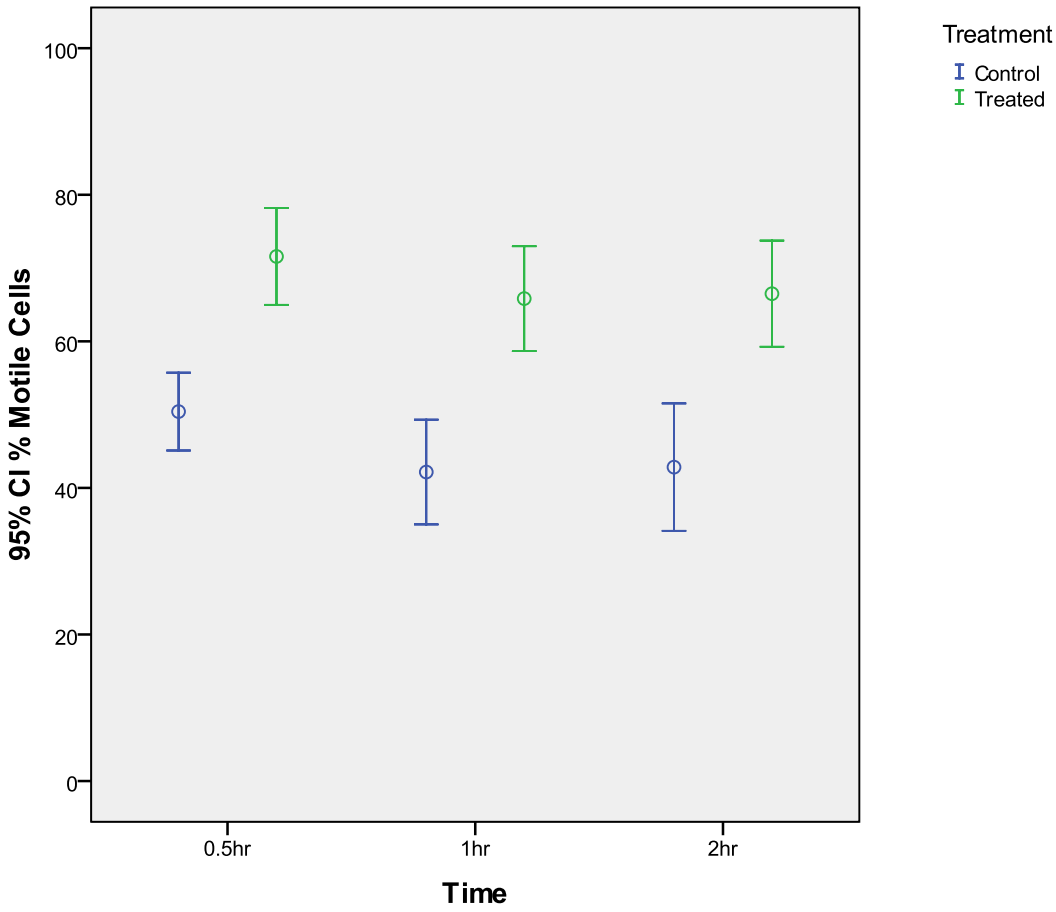

Figure 5.5 Each bar represents the mean percent of motile cells with or without the addition of Papaverine on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

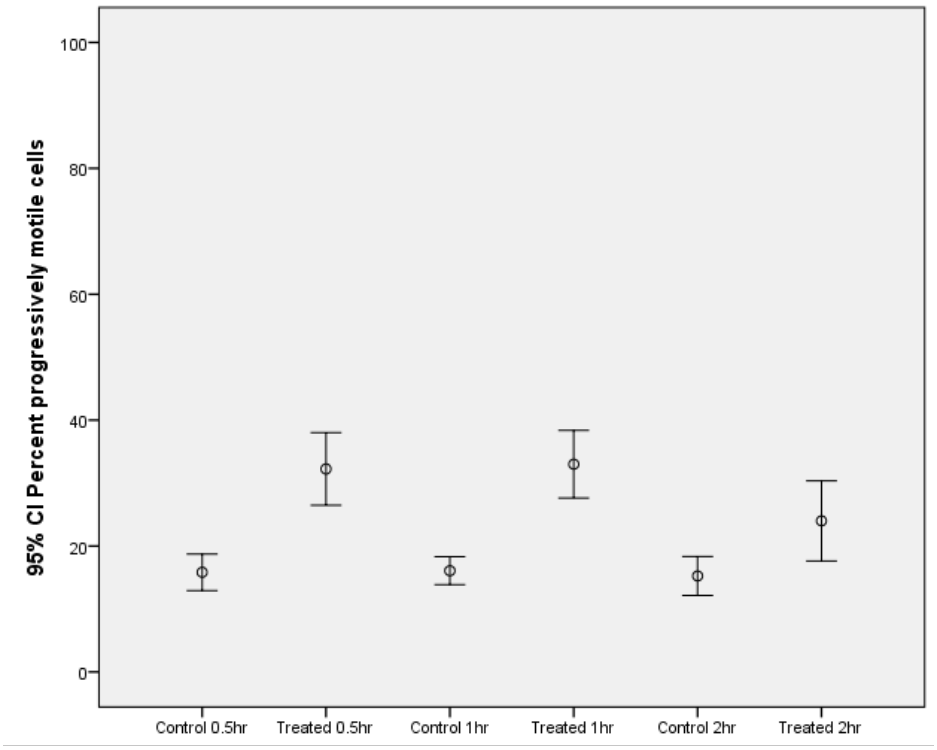

Figure 5.6 Each bar represents the mean percent of progressively motile cells with or without the addition of Papaverine on the 40% of three different patient samples at 0.5, 1 and 2 hour incubation periods.

Table 5.1 gives a comparison of the effects of different PDE inhibitors (including those used in previous sections) on human sperm motility. This is demonstrated after 1 hour sperm-PDE inhibitor incubation as this appears to be when PDE inhibitors have the greatest effect on sperm motility. The inhibitor showing the greatest effect on the 40% fraction of patient sperm samples is PDE-7 type specific BRL ($P<0.05$). However, all five of the PDE inhibitors investigated have shown a significant increase in the mean percent of motile sperm cells with the 40% fraction of a patient population.

PDE Inhibitor	PDE Inhibited	Sample	Percent motile Mean ± SEM	P-value
8-MeOM-IBMX	PDE-1	C T Difference	16.5 ± 9.0 32.6 ± 13.4 16.1 ± 4.4	0.036
Rolipram	PDE-4	C T Difference	28.8 ± 11.3 45.6 ± 9.9 16.8 ± 1.4	0.02
Milrinone	PDE-3	C T Difference	42.2 ± 10.6 66.1 ± 8.8 23.9 ± 1.8	0.008
BRL	PDE-7	C T Difference	42.2 ± 10.6 71.3 ± 10.0 29.1 ± 0.6	0.0004
Papaverine	PDE-10	C T Difference	42.2 ± 10.6 65.8 ± 12.3 23.6 ± 1.7	0.005

Table 5.1 A comparison of the effects of various PDE inhibitors on sperm motility of the 40% fraction of three different patient sperm samples after 1 hour incubation.

These results demonstrate the consistency of a significant difference between the percent of motile cells of patient sperm with or without PDE inhibitors. This is demonstrated particularly 1 hour after the addition of BRL. Due to these results, a wash step was carried out using BRL on the 40% fraction of three different patient samples. Samples were prepared as previously described (Chapter 3 3.4.2) and BRL was added at a final concentration of 100µM. This was done to determine whether BRL can maintain the positive effects demonstrated after 1 hour incubation even after an additional wash step. T-test was used in statistical analysis and $P<0.05$ was considered significant.

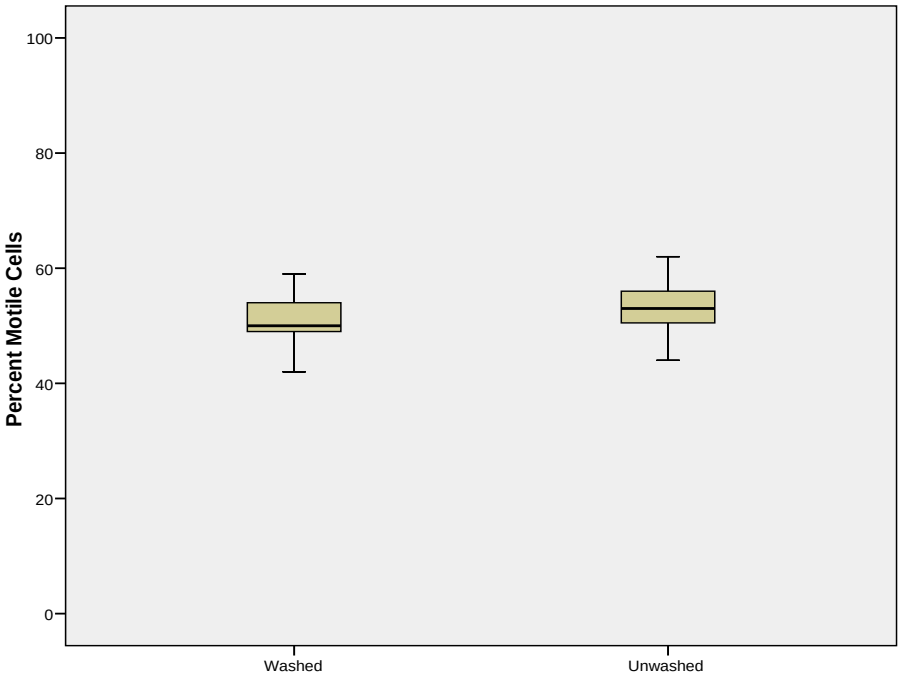

Figure 5.7 Graph demonstrates the mean percent of motile cells in the 40% fraction of three different patient sperm samples with or without the addition BRL after 1 hour incubation with or without further density centrifugation.

There was no significant difference in the mean percent of motile cells incubated for 1 hour with BRL with or without an additional wash step ($P>0.05$).

5.4 Discussion

The results demonstrate a significant increase in the percent of motile cells in patient sperm samples using Milrinone, BRL or Papaverine.

Milrinone has been previously studied in the past with regards to the percent of hyperactivated and capacitated cells and was shown to have no effect on sperm functions (Lefievre et al., 2002). The results in this study however suggest that Milrinone increases the percent of motile sperm in patient samples. However, the samples used in the study by Lefievre et al 2002 were carried out on healthy volunteers unlike the results of this study which are based on sub-fertile patient samples. As discussed in other chapters, it may be that the motility from samples of healthy donors cannot be enhanced unlike sperm from patient samples where there is room for improvement. This suggests a target population of sub-fertile men.

Papaverine also demonstrated a significant increase in the percent of motile sperm of patient samples in this study. Previously the effects of Papaverine have been investigated and have shown to have a significant effect on human sperm (Torres-Flores et al., 2009). However, Torres-Flores et al 2009 investigated levels of cAMP, PKA and Ca^{2+} with the addition of Papaverine. These factors which are closely linked with sperm motility, enhanced with the addition of Papaverine. They enhanced to such an extent that they were higher than the levels of what PTX (used as a positive control) can provoke. However, this study did not investigate the direct impact of Papaverine on the percent of motile cells present in a sample. Again,

Torres-Flores et al 2009 carried out this study using healthy donors which cannot be a comparison for samples with poor motility. The results gained here were from three sub-fertile patients. This gives a representation of the effect of using PDE inhibitors in a clinical setting.

There was also a significant increase in the percent of motile cells when sperm were incubated with BRL. To date, no studies have been carried out using BRL on sperm. The importance of these results includes the fact that this is new information with regards to the effect of PDEs on patient sperm. These preliminary results emphasise the importance of further investigation using PDE inhibitors to overcome the present limited knowledge of PDE inhibitors and human sperm. This PDE inhibitor also maintained its effect on enhancing the percent of motile cells even after a wash stage. The ability of the PDE inhibitor to maintain its effects suggests a clinical advantage of the use of BRL in ART as discussed previously (Chapter 3 3.4).

The results gathered in this thesis make it even more surprising that little is known about the effects of PDE inhibitors on sperm motility and function. The results from this alongside studies previously carried out have demonstrated a strong correlation between PDE inhibitors and an improvement in sperm motility. For this reason it is important to investigate this further, particularly since the results demonstrated in this study involve patient sperm samples. The low number of patient samples used in the previous sections are only enough to gain a sense of what effect PDE inhibitors have on sperm motility. The next chapter investigates the effect of PDE inhibitors on

a larger sperm population in order to gain a better understanding of the percent of the population of sub-fertile men which PDE inhibitors have an effect on.

Chapter 6: Screening patient samples

6.1 Introduction

Experiments carried out previously in this study have included relatively low sample numbers in order to gain an insight into the effect of PDE inhibitors on human sperm motility and an indication as to whether motility can be improved. The purpose of this chapter was to determine the percent of a sub-fertile male population for each IVF and ICSI, which PDE inhibitors have an effect on. Sperm parameters can vary dramatically on an individual basis, even between samples from healthy donors (Keel, 2006). The aim of this section was to overcome this insufficiency by investigating the effect of PDE inhibitors on larger populations of sub-fertile males attending ACU Dundee for either IVF or ICSI. In doing so, it was hoped that a better understanding may be gained of the extent in which PDE inhibitors improve sperm motility. The percentage of progressively motile sperm cells was also analysed in this section. Progressive motility is an important factor of sperm motility as it enables sperm to penetrate cervical mucus (as discussed in next chapter) and in turn reach the oocyte. It is known that fertilisation rates are dependent upon progressively motile sperm (Simon and Lewis, 2011) and the assessment of progressive sperm is more clinically relevant than slow moving sperm (Lars, 2010). The presence of sperm with substantial forward progression is naturally important, whereas clinically this has been overcome by ICSI. If PDEs were used clinically with an aim to reduce cost for patients and convert what would be ICSI samples now suitable for IVF or IUI after PDE inhibitor incubation, it is important to know that the sperm cells being used for

the procedure are of fertilising capability. This is hugely important due to the risk of fertilisation failure by converting samples which would have undergone manual injection to IVF or IUI where sperm must somewhat naturally fertilise the oocyte. This section however makes the distinction between slow and rapid cells in IVF and ICSI populations by analysing the percentage of progressively motile cells. PDE inhibitors Rolipram and BRL were used in this section. Reasons for this include Rolipram which is specific to PDE-4 has already been studied in sperm and the aim of this was to compare results with studies previously carried out. BRL is used in this section as there is no evidence of BRL being used with sperm and thus any result here would be novel.

6.2 Materials and Methods

ICSI sperm sample preparation

Semen samples from 27 patients consented for research in ACU Dundee were collected and prepared within 30 minutes of production by an embryologist. The sperm samples were examined including semen volume, concentration and motility parameters on the raw semen sample. A maximum of 2.6ml sample was layered on a 40%/80% PureSperm® density gradient in a 5ml sterile round bottom falcon tube. Samples were then centrifuged at 300g for 20 minutes. After centrifugation the supernatant was removed with a sterile 5ml falcon glass pipette and the pellet removed using a sterile pasture pipette into a sterile 5ml round bottom falcon tube

filled with 4ml COOK Sydney IVF Gamete Buffer (SIGB). The pellet was then centrifuged at 500g for 10 minutes as a wash step. The media was then removed down to roughly 300µl leaving the pellet suspended in the SIGB. A concentration check was carried out under Inverted System Microscope Olympus ix51 by simple eyeball observation to ensure motile sperm had been recovered. The pellet was then left at room temperature conditions until ICSI. After the injection, the sample was used in this study. It is the 80% fraction of patient sperm sample that is used in this section.

IVF sperm sample preparation

Samples were collected from 33 patients and assessed as above by an embryologist. Prior to preparation of IVF suitable sperm, the media used for sperm wash also known as Sydney IVF Sperm Media (SISM) is pre-equilibrated overnight at 37°C, 6% CO_2. Sperm samples were prepared as above using SISM in place of SIGB. Following the wash the pellet was then suspended in up to 1ml of SISM. At this stage, another sperm assessment was carried out including sperm concentration using a Neubeaur chamber which at this point is critical in the calculation for IVF insemination. This suspension was hand gassed for approximately 20 seconds using a sterile Hunter pipette fitted to the rubber tubing of a 6% CO_2 in air gas cylinder. This was left at room temperature with the lids sealed until 1 hour before insemination where the lids were loosened and samples were placed in a 37°C, 6% CO_2 incubator for 1 hour. After insemination, samples were used in this study. It is the 80% fraction of patient sperm sample that is used in this section.

Sperm-PDE inhibitor incubation

After sperm preparation, 99µl of either ICSI or IVF sperm suspensions were placed in eppendorfs and 1µl of either Rolipram or BRL at final (concentrations of 10µM and 100µM respectively) were added to the samples. The control contained no PDE inhibitor. For sperm-PDE inhibitor incubation, IVF samples were placed in a 37°C, 6% CO_2 incubator whereas ICSI samples were left at room temperature conditions. The mean percent of motile cells and the mean percent of progressively motile cells were recorded with CASA at 1 hour post PDE inhibitor addition as this seems to be when PDE inhibitors have the greatest effect. T-test was used in statistical analysis and P value <0.05 was considered significant.

6.3 Results

There was no significant difference in the mean percent of motile cells between control and Rolipram treated 80% fraction of IVF samples after 1 hour incubation (mean 77.2% and 75.8% respectively $P>0.05$) (Figure 6.1). There was however a significant increase in the percent of motile cells between control and BRL treated IVF samples after 1 hour incubation (mean 77.2% and 85.9% respectively $P<0.05$). The percent of the population of sub-fertile men treated for IVF which were positively effected by the use of Rolipram or BRL was 53.3% and 63.3% respectively. These figures were derived from raw data.

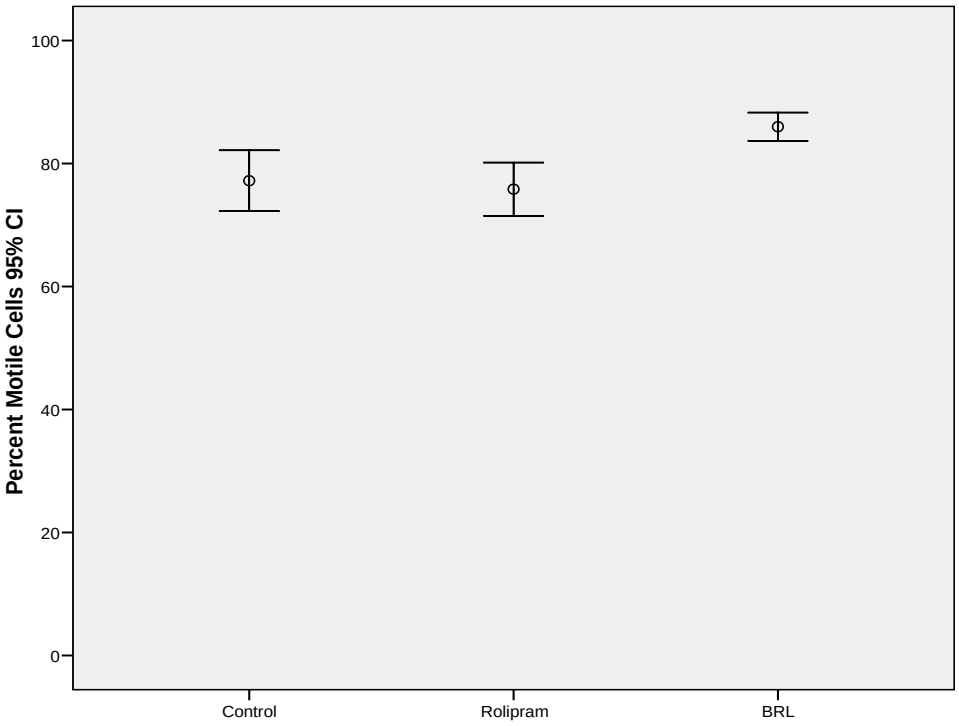

Figure 6.1 Graph demonstrates the mean percent of motile cells from the 80% fraction of 33 IVF patient sperm samples with or without the addition of Rolipram or BRL after 1 hour incubation period.

There was a significant difference in the mean percent of motile cells between control and treated following incubation with Rolipram in ICSI samples after 1 hour incubation (mean 14.8% and 39.4% respectively $P<0.05$) (Figure 6.2). There was also a significant difference in the mean percent of motile cells between control and treated following incubation with BRL in ICSI samples after 1 hour incubation (mean 14.8% and 32.0% respectively $P<0.05$). The percent of the population of sub-fertile men treated for ICSI positively effected with the use of Rolipram and BRL was 96.7% and 98.3% respectively. These figures were derived from raw data.

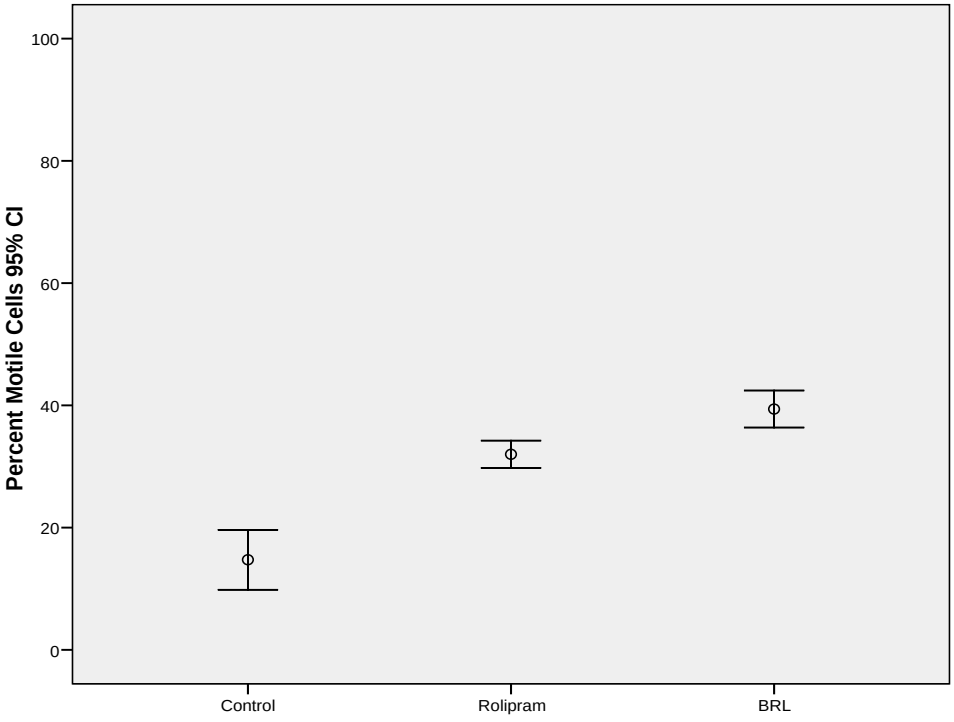

Figure 6.2 Graph demonstrates the mean percent of motile cells from the 80% fraction of 27 ICSI patient sperm samples with or without the addition of Rolipram or BRL after 1 hour incubation period.

There was no statistically significant difference ($P>0.05$) in the mean percent of progressively motile sperm in the 80% fraction of the IVF population when incubation with either Rolipram (Figure 6.3) or BRL (Figure 6.4). However there was a statistically significant increase ($P<0.001$) in the percent of progressively motile sperm in the 80% fraction of the ICSI population when samples were incubated with either Rolipram (Figure 6.5) or BRL (Figure 6.6).

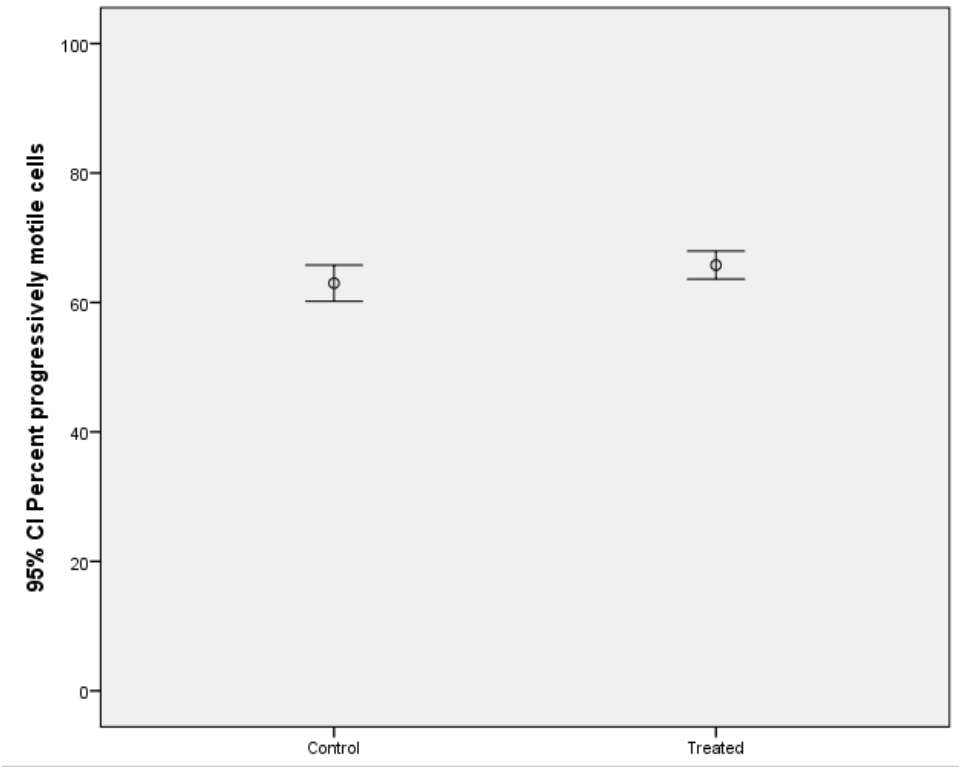

Figure 6.3 Graph demonstrates the mean percent of progressively motile cells from the 80% fraction of 33 IVF patient sperm samples with or without the addition of Rolipram after 1 hour incubation period.

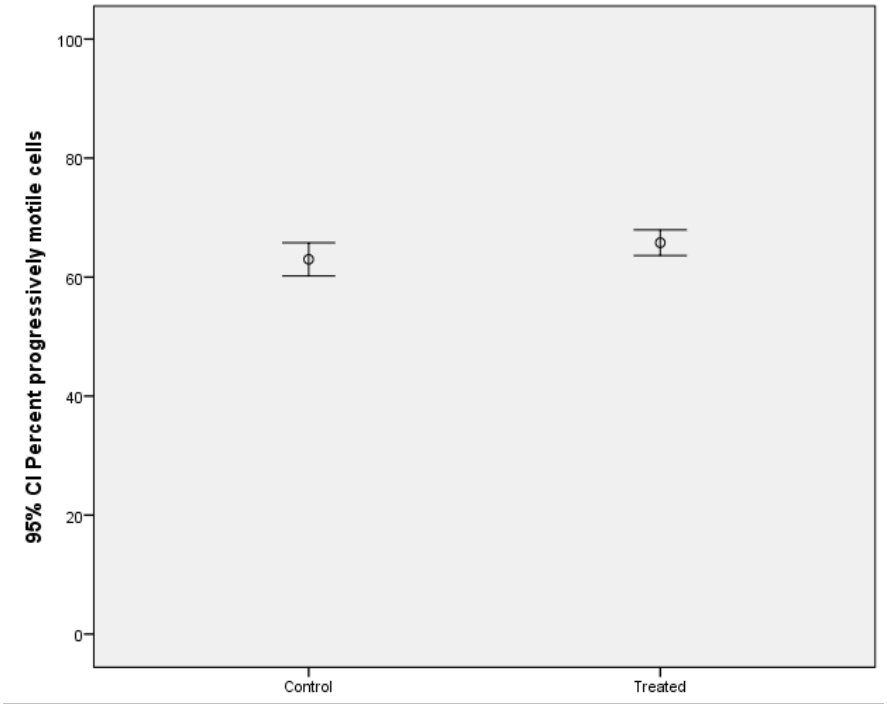

Figure 6.4 Graph demonstrates the mean percent of progressively motile cells from the 80% fraction of 33 IVF patient sperm samples with or without the addition of BRL after 1 hour incubation period.

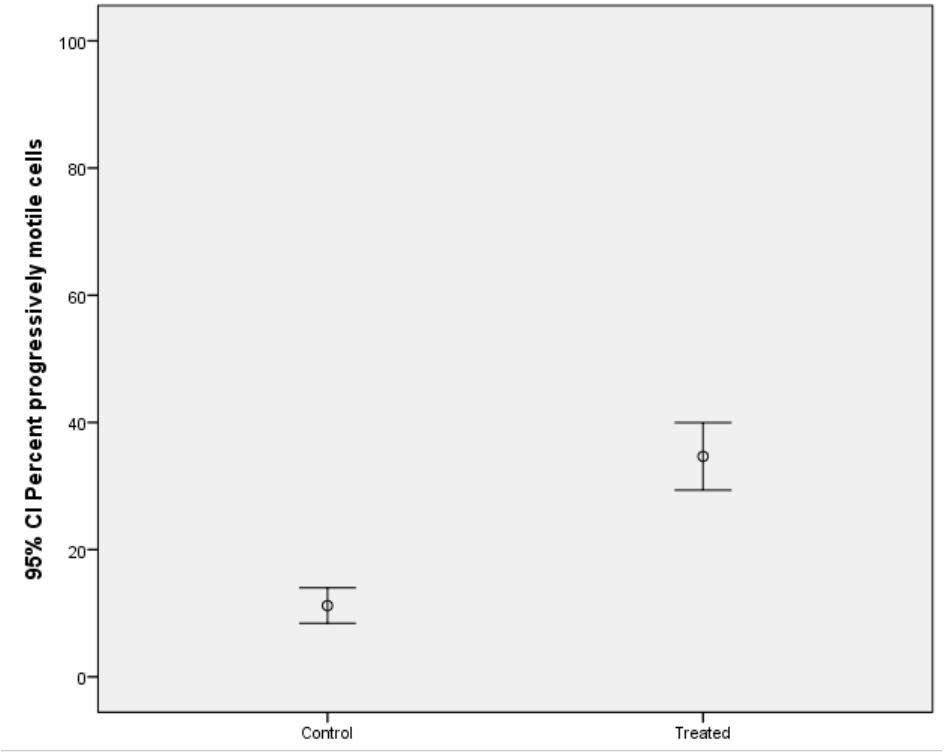

Figure 6.5 Graph demonstrates the mean percent of progressively motile cells from the 80% fraction of 27 ICSI patient sperm samples with or without the addition of Rolipram after 1 hour incubation period.

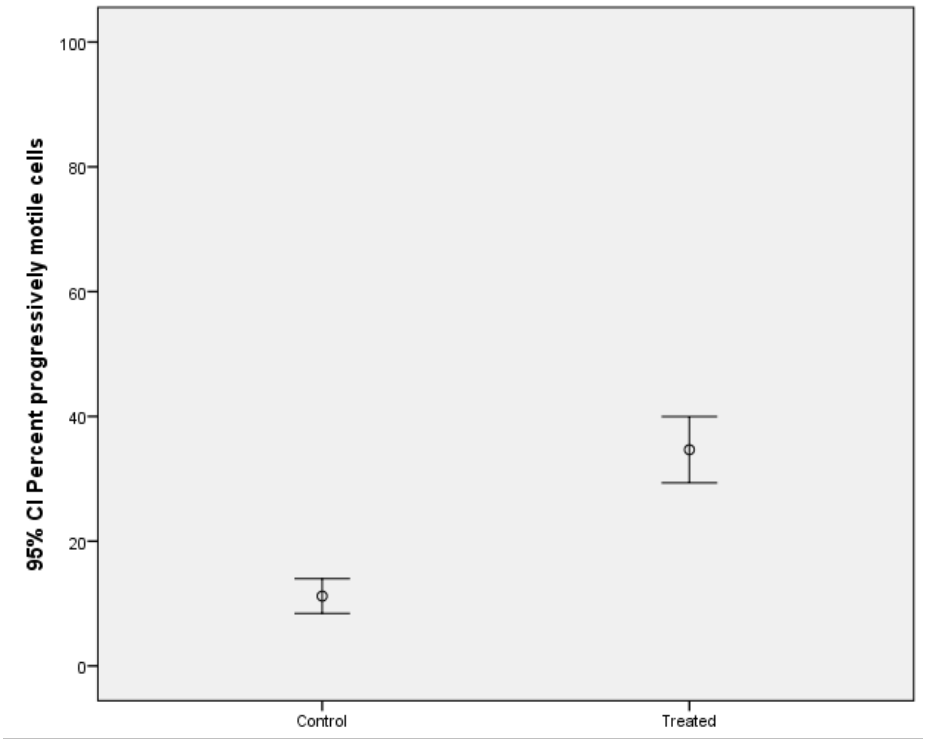

Figure 6.6 Graph demonstrates the mean percent of progressively motile cells from the 80% fraction of 27 ICSI patient sperm samples with or without the addition of BRL after 1 hour incubation period.

6.4 Discussion

In conclusion to this section, sperm quality of most IVF semen samples is normal. Patients attending for IVF treatment are of female factor infertility including common causes such as tubular disorders and endometriosis. The sperm sample from these couples is generally normal and would probably be IUI if not for the female infertility factor. This may therefore be the reason why the effect from incubation with PDE inhibitors on IVF sperm (if any) was limited. Patients attending for ICSI

treatment are however mainly male factor infertility (as discussed in Chapter 1). The low percent of motile cells may mean that there is more room for improvement, hence the dramatic increase in percent of motile cells when sperm are incubated with PDE inhibitors. The results suggest that PDE inhibitor addition is mainly suitable for poor quality sperm, in other words, those attending for ICSI and this is most likely the target population.

Based on the WHO criteria, previous studies have mainly examined samples with normal semen analyses (Levin et al., 1981; Rees et al., 1990). However, data from this thesis supports the study by Yovich et al 1990 suggesting that PDE inhibitors may be suited for those with poorer semen analysis. Also supporting this, Tash and Means 1983 suggest that highly motile sperm may have a maximally stimulated protein kinase A. In turn, with the use of PDE inhibitors an increase in cAMP may have no additional effect. This may be concluded by results from thesis where PDE inhibitors were added to normal semen samples and there was no change in motility (Chapter 2 2.3.1) in contrast to where PDE inhibitors were added to abnormal semen samples demonstrating a significant increase in sperm motility (Chapter 2 2.3.2).

Since 11 PDE families now exist, it may be postulated that some are in the sperm cell. Selective inhibition of PDEs has allowed enhancement of sperm motility as the previous results have demonstrated. While all PDE types have not been identified in human sperm, these results show the possible existence of at least five distinct PDE forms. This conclusion is supported by this PDE inhibition study when considering the percent of each (IVF and ICSI) population is actually effected by the drugs.

During inhibition by PDE type specific inhibitors, sperm motility increased. The various PDE subtypes (3, 7 and 10) may be located within the human sperm. With regards to PDE types 1 and 4, these have already been located within the human sperm cell (Fisch., et al 1998). It may therefore be possible that other PDE types may be present from these results of sperm activity.

Lastly, it is important to consider the effect of PDE inhibitors on the progressive sperm count. The percent of progressively motile cells in the ICSI population dramatically increased when sperm were incubated with either Rolipram or BRL. These results are of great clinical significance since progressive motility is mainly what determines the required procedure for individual samples. For instance, a sample of mainly slow moving sperm following preparation would most likely be ICSI whereas samples containing a high amount of progressive sperm would be IUI (or IVF where female factors contribute to infertility). It is therefore not surprising that samples of progressively motile cells have been strongly correlated with fertilisation and pregnancy rates (Donnelly et al., 1998). The results from this section therefore suggest that in increasing sperm motility of samples from sub-fertile men, PDE inhibitors may have a positive effect on sperm function. The next chapter explores the effect of PDE inhibitors on the sperm-oocyte interaction.

Chapter 7: Investigating the effects of PDE specific inhibitors on human sperm function

7.1 Sperm penetration test

7.1.1 Introduction

The interaction between human sperm and cervical mucus is one of the best diagnostic tools of male infertility. It has been long known that the functional capacity of human sperm in vitro can serve as a better tool in predicting fertility than the measurement of traditional semen parameters (Hull et al., 1984; Barratt et al., 1989; Biljan et al., 1994). Sperm motility is the single most important characteristic which should allow penetration and migration within cervical mucus (Mortimer, 1983). In a normal semen sample, the propulsive forces generated by sperm motility thrust the sperm through cervical mucus. The function of cervical mucus is to filter out sperm with poor morphology and motility, acting as a barrier to abnormal sperm. This is the case for every semen sample, however more so in those with a higher abnormal sperm count. Sperm from samples of an asthenozoospermic nature may find penetration of cervical mucus difficult due to the lack of progressive propulsion (as demonstrated in the ICSI population, Chapter 6 6.3). This is required to enable sperm to the vicinity of the oocyte. The difference in the ability of sperm to penetrate viscous substances has been previously demonstrated (Barratt et al., 1989). This study demonstrated a clear difference between two groups of men (56 semen samples in total) with regards to sperm penetration ability with those failing to penetrate

cervical mucus also failed to penetrate an oocyte. This study also demonstrated the predictive value of the human cervical mucus test (HCM) between fertile and infertile men since some semen characteristics were normal yet those sperm failed to penetrate cervical mucus and also failed to fertilise an oocyte. This confirmed other studies carried out showing positive relationships between in vivo penetration of human cervical mucus and in vitro fertilisation of human oocytes (Hull et al., 1984). Testing sperm function in vitro is therefore a valuable predictor of fertilisation results.

As previously demonstrated, PDE inhibitors do have an effect on sperm motility. From this it would be reasonable to assume that PDE inhibitors may also affect sperm function. There is limited information regarding the effects of PDE inhibitors on sperm function. Nassar et al 1999 used PTX to investigate its effect on human sperm motility and bovine cervical mucus penetrability (BCMP). They observed a higher BCMP score with asthenozoospermic samples but not with normozoospermic samples, again demonstrating the predictive value of this test and demonstrating a positive correlation with the BCMP score and samples incubated with PTX. PTX enhanced sperm function allowing more sperm to penetrate bovine cervical mucus suggesting a potential use of PDE inhibitors for sperm with motility defects. This however, seems to be the only study available with information on the effect of PDE inhibitors on sperm function.

Traditional semen parameters (mainly concentration, motility and morphology), have provided diagnostic information and have predicted fertilisation rates over the years.

Although the conventional semen analysis is critical as a tool to diagnose infertility, the information gained is limited. Various sperm tests have been investigated to determine alternative diagnostic steps in ART including the sperm-cervical mucus penetration test. With the addition of PDE type specific inhibitors, sperm-cervical mucus tests may not only be a diagnostic tool but a solution for asthenozoospermia samples to overcome the issue of reaching the oocyte.

In turn, motility plays an important role in fertilisation by promoting binding of the sperm to the zona pellucida and initiation of the acrosome reaction. Motility is therefore key in the recognition stage as it provides interaction between gametes. If poor quality sperm are then adapted in such a way in which they are enabled to reach the egg then the fertilisation potential of poorer semen samples has already been enhanced. By the use of PDE inhibitors, increasing motility of sperm would enhance the fertilisation potential by increasing the not only the number of sperm at the site of fertilisation, but also the number of progressively motile sperm which are more able to penetrate viscous media. The next crucial step in predicting successful fertilisation is to analyse the journey of the sperm to the oocyte.

7.1.2 Materials and Methods

The 80% fraction of 15 patients attending for ICSI treatment was used in this study. Semen samples were prepared as described previously (Chapter 6 6.2). Eppendorfs of control and treated containing penetration media were prepared (Methylcellulose

[MC] at a viscosity of 4000cp, prepared in 10mg/ml in NCB containing 0.45% BSA [0.0045g/ml BSA]). Separate eppendorfs of control and treated were then prepared and the suspended sample divided between them (~100µl per eppendorf) adding the PDE inhibitor Rolipram or BRL to the 'treated' eppendorf. Both were covered with parafilm to prevent the sample evaporating. Long flattened capillary tubes with a length of 5cm and a depth of 0.4mm were placed into each eppendorf containing penetration media. Once the tube has filled and excess media wiped from the tubes one end of the capillary tube was sealed with plasticine and a distance of 1cm marked on the opposite side. The open ends of the capillary tubes were placed in the eppendorfs containing the prepared sperm, piercing through the parafilm. Both the control and treated eppendorfs were left in 37°C 6% CO_2 conditions to allow sperm to migrate. A 1 hour incubation period was chosen due the nature of the previous results demonstrating that various PDE inhibitors have the greatest effect at this time point. The capillary tubes were kept vertical to allow sperm to migrate against gravity, mimicking the effects of the female tract. The capillary tubes were then removed from the semen samples and wiped to remove residual spermatozoa from the surface of the glass. A sperm count was taken using CASA from the 1cm mark on the capillary tubes. Readings were taken from 4 fields per plane with a total of 4 planes. T-test was used in statistical analysis and P value <0.05 was considered significant.

7.1.3 Results

With the addition of either Rolipram or BRL there was a significant increase ($P<0.05$) in the mean cell number at the 1cm mark on the glass capillary tube (Figure 7.1.1) from the 80% fraction of ICSI patient sperm. The mean number of cells without PDE inhibitor, treated with Rolipram and treated with BRL was 47.5, 145.7 and 149.7 respectively.

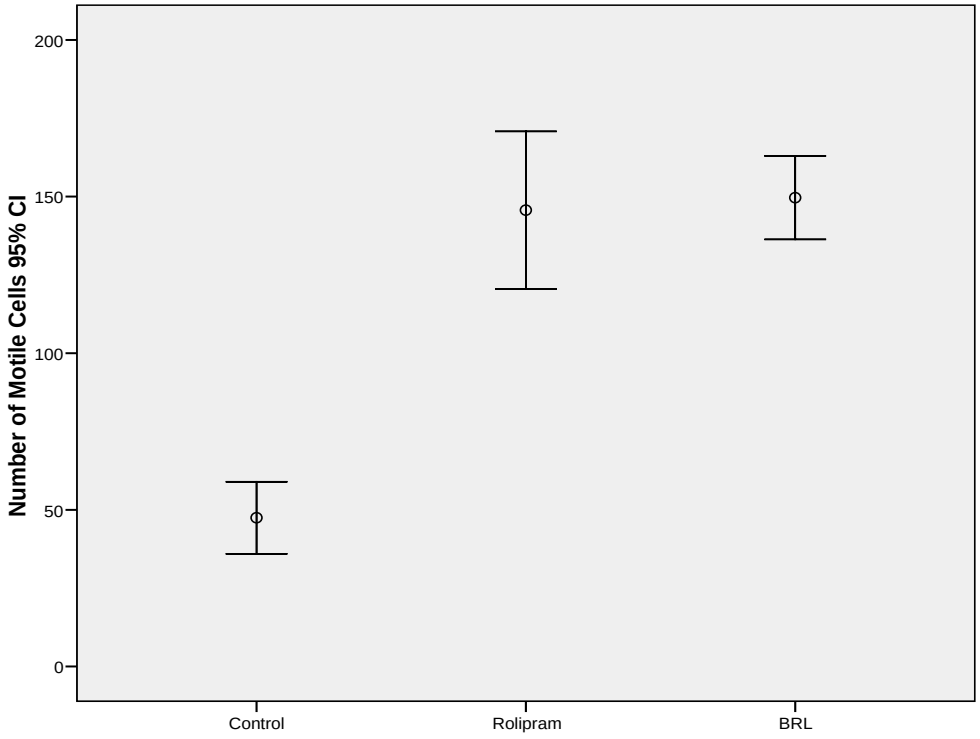

Figure 7.1.1 Graph demonstrates the mean number of motile cells at the 1cm mark of glass capillary tubes after the 80% fraction of 15 ICSI patient sperm samples were incubated with Rolipram, BRL or neither.

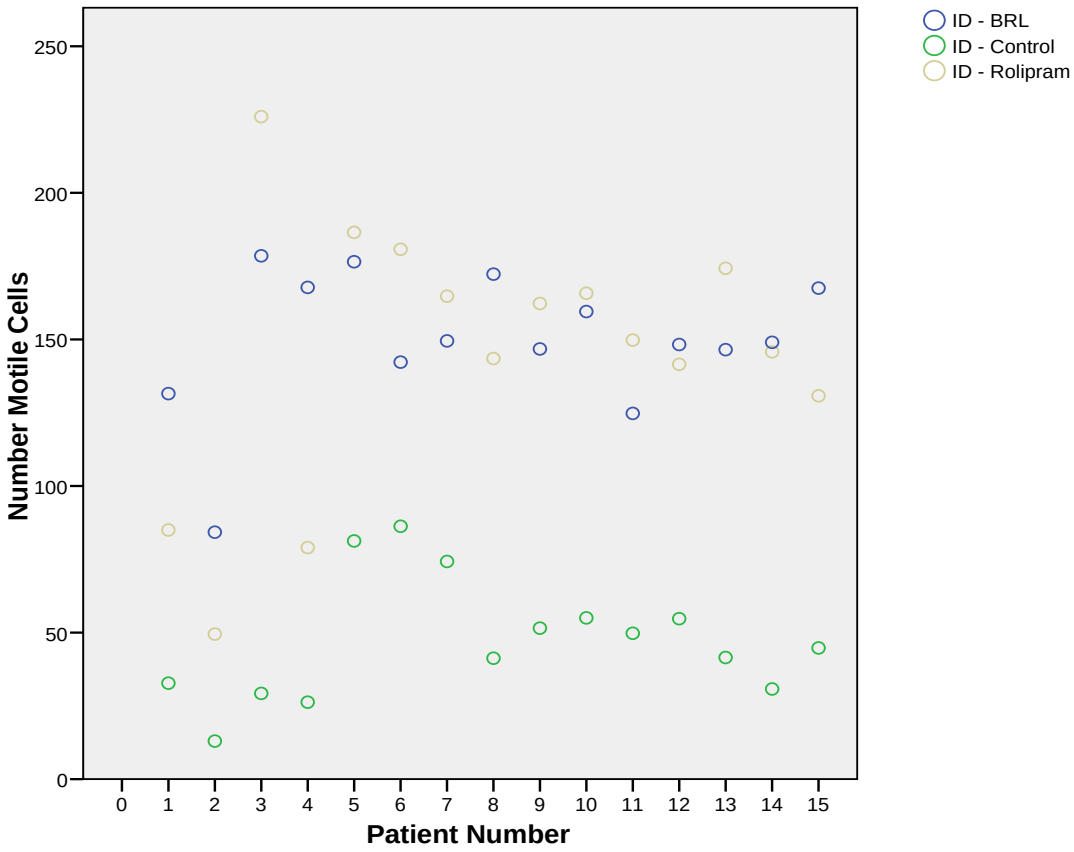

Figure 7.1.2 Scatter plot demonstrating the number of motile cells from the 80% fraction of 15 ICSI patient sperm samples is significantly higher in both Rolipram and BRL treated samples when compared with control.

The results are further emphasised in figure 7.1.2 showing the number of motile cells is considerably higher for each individual patient when treated with Rolipram or BRL.

7.1.4 Discussion

The sperm penetration test is a well-known and well developed method of testing sperm function. It is a predictor of fertilisation outcome through monitoring the distance a sperm can travel through a cervical mucus type substance (Barratt et al., 1989; Ivic et al., 2002). With the addition of PDE inhibitors a comparison can be made on how sperm react to this activity. The above results demonstrate that when PDE inhibitors are added to an ICSI sperm suspension the effects are significant. A significantly greater number of sperm reach the proposed mark when incubated with a PDE inhibitor than without. When put into a clinical setting this suggests that sperm which have been incubated with the drug have a greater chance of reaching the oocyte in vivo than those without. For this to have such an effect on samples which are suitable for ICSI, it may resort to them being converted to IVF or even IUI if the reason for infertility is predominantly male factor. Overall, this experiment was a useful method of determining the fertilisation potential of sperm with or without PDE inhibitor. It would appear that not only are PDE inhibitors beneficial for enhancing patient sperm motility, they also have a positive effect on sperm function by allowing more sperm to penetrate a cervical mucus like substance and presumably reach the oocyte. However, although PDE inhibitors appear to have a beneficial effect on patient sperm motility and in addition to that, sperm function, it is completely unknown as to the effect they would have as sperm reach and interact with the oocyte.

7.2 Human sperm-zona pellucida binding test

7.2.1 Introduction

Sperm-zona pellucida binding is the best predictor of IVF outcome as it is the first crucial step of sperm-oocyte interaction (Oehninger et al., 1992). On binding to the oocyte, the zona pellucida acts as an agonist and enables the sperm to undergo physiological changes such as the acrosome reaction. With propulsive forces and hyperactivation, sperm penetrate the zona pellucida allowing fertilisation to proceed. Samples of an abnormal nature however i.e. morphologically abnormal or those with a low percentage of progressively motile cells cannot generate these propulsive forces required during sperm-oocyte interaction. It has long been known that sperm motility is significantly correlated with in vitro fertilisation rates (Liu et al., 1991). Reasons for couples attending an assisted conception unit for ICSI include low sperm count, high proportion of morphologically abnormal sperm and mainly, due to lack of progressive sperm cells. For a small proportion of patients, the reason for having ICSI is in fact due to a previous complete fertilisation failure with conventional IVF. Roughly 5% of all IVF cases experience failed fertilisation and when the reason is explained to the patient as unknown, this may in fact be an issue with sperm binding to the zona pellucida.

Sperm-zona pellucida binding tests have been carried out since the late 1970s using hamster, pig and human oocytes (Yanagimachi et al., 1979; Green, 1988). The reason for such experiments at this time was to gain an understanding of the biological

characteristics of the zona pellucida. This is shown in studies such as Tesarik et al 1988 which investigated the difference in human zona pellucida resistance to sperm penetration using oocytes at various stages of maturation. Studies such as this are the foundation for our knowledge of the regulation of sperm-oocyte interaction. It was at this time that the sperm-zona pellucida interaction was suggested to be a contributing factor to unexplained human infertility (Trounson et al., 1980). Today this remains the same as demonstrated by Liu et al 2011. This study demonstrated a lower number of human sperm bound to the zona pellucida in a group of patients with unexplained infertility than those with female factor infertility. The fertilisation rate for the unexplained infertility group was also lower than that of female factor. This shows a positive relationship between sperm-zona pellucida binding and fertilisation rate. In turn, the sperm-zona pellucida binding test is a strong suggestion as a clinical sperm function test for patients with unexplained infertility to avoid complete fertilisation failure.

Liu, et al. 2011 also discussed the importance of the acrosome during sperm-oocyte interaction. They correlated fertilisation rate with induced acrosome reaction where the group with the highest number of acrosome reacted sperm also had the lowest fertilisation rate. Propulsive forces and hyperactivation are both important factors of motility and are crucial for sperm to bind to the zona pellucida. Acrosome reacted sperm are an indicator that sperm-zona pellucida interaction has taken place. However, premature acrosome reacted sperm cannot fertilise (Liu and Baker., 1990) and considering hyperactivated sperm are more likely to acrosome react (Liu et al., 2007) it is a complete unknown as to the effect any PDE inhibitor would have on this

function. On the other hand, acrosome reacted sperm can still penetrate the zona pellucida, providing the sperm are in the vicinity of the oocyte. The problem therefore lies in defining sperm-zona pellucida binding. Sperm are bound due to the fact that the acrosome reaction has taken place, leaving loosely bound sperm to be easily unattached from the zona pellucida. Regardless of the definitive, the conclusive association of the sperm and the oocyte depend on clinical observations at fertilisation checks. With the use of a PDE inhibitor it is possible that poor quality sperm may not only be able to reach the oocyte, but more importantly, bind to the zona pellucida.

Two bioassays have been developed since the late 1980s known as the hemizona assay (HZA) and the sperm-zona binding ratio test, both providing clinical information on sperm binding to the zona pellucida in fertile and infertile couples. The HZA comprises of two functionally equal zona halves which have been microsurgically split. The value of this test is that is provides a comparison between the two halves of sperm bound to the same oocyte surface. The test can also be performed on a single oocyte which aids the problem of limited oocytes available for research. This assay has long been used as a clinical predictor of fertilisation rates, identifying those patients likely to encounter fertilisation problems during IVF (Franken et al., 1989). The other sperm-zona pellucida binding test uses oocytes which have failed to fertilise during IVF. With two different sperm populations (fertile and infertile) labelled with fluorescent dye, they are mixed with a group of oocytes and incubated. Oocytes are then assessed for tightly bound sperm. The problem with this type of study is the high number of oocytes required. Below is an

example of a single sperm cell binding to the zona pellucida of a Metaphase 1 oocyte (Figure 7.2.1). Here the sperm head can clearly be seen as it makes contact with the zona pellucida. This depicts the initial stages of sperm-oocyte interaction and is one of the most important steps in human fertilisation.

Figure 7.2.1 Sperm-zona pellucida binding. The initial stages of sperm-oocyte interaction.

This final section of the study aims to determine the effect of PDE inhibitors on sperm-oocyte interaction by investigating sperm-zona pellucida binding after ICSI patient sperm are incubated with Rolipram. The creation of this technique was inspired by personal experience in ACU Dundee which provided a sense of clinical application for this study.

7.2.2 Materials and Methods

Human oocytes were obtained through consenting patients in the ACU Ninewells Hospital attending oocyte collection prior to ICSI treatment only. Those undergoing IVF were not used in this study as the oocytes are not assessed prior to insemination and all oocytes are used in treatment. Patients suitable for ICSI were also chosen as immature oocytes are identifiable on the day of oocyte collection and not used for injection. Oocytes which were not suitable for treatment i.e. germinal vesicle (GV) and metaphase 1 (M1) stage oocytes, were therefore kept aside for experimental purposes and used in this study fresh on the day of oocyte retrieval.

After the Embryologist performing the ICSI procedure selects the oocytes suitable for ICSI, GV and M1 oocytes were left in 5µl SIGB drops with a 3.0ml overlay of Sydney IVF culture oil (SICO) in the ICSI injection dish at 37°C. An ICSI dish was equilibrated at 37°C, 6% CO_2 for 4 hours containing the required number of 5µl drops of SIFM, some of which were wash drops and others were control and treated drops which will eventually contain the sperm and dissected zona pellucida. This was covered with a 3.0ml SICO overlay.

During dish equilibration, the sperm suspension from the male partner was used after the ICSI procedure was carried out. The sperm already suspended in approximately 0.3ml of SIGB was made up to 1ml of SIGB kept at room temperature and a sperm count was taken with a Neubeaur chamber to determine the concentration required (20M/ml standard). This was then centrifuged at 300g for 5 minutes to gain a pellet.

The SIGB after the wash was taken off using a glass pipette and the pellet was suspended in up to 1ml of hand gassed (6% CO_2) SISM. This was left for 3 hours at room temperature for the sperm to initiate capacitation prior to zona-binding, mimicking our clinical IVF procedure. Following this, the sperm suspension was centrifuged at 300g for 5 minutes and suspended in SIFM. Two eppendorfs (control and treated) from this sperm suspension were prepared, each containing 99µl of the sperm suspension with one containing 1µl of Rolipram at a final concentration of 10µM. The control and treated SIFM drops within the dish were then taken out and replaced with 5µl of each the control and treated samples from the eppendorfs.

GV and M1 oocytes were dissected in their injection dish using insulin needles. The ooplasmic contents of the oocytes were released and the empty zonas were split as close to equal halves as possible (Figure 7.2.2). The zona halves were then taken through the wash drops within the equilibrated dish to remove HEPES based media and each half was placed in the sperm-SIFM drops. The dish containing both sperm and zona pellucida was left in 37°C, 6% CO_2 conditions for 2 hours giving the sperm sufficient time to bind to the zona pellucida. After this time the zona pellucida halves were aspirated through more wash drops to remove loosely bound sperm. Whilst in the last wash drops the dish was placed on the stage of Inverted System Microscope Olympus ix51 and observed at 40x magnification (Figure 7.2.3). Images were obtained through the computer. Wilcoxon matched pairs signed ranks test was used in statistical analysis and a P value of <0.05 was considered significant.

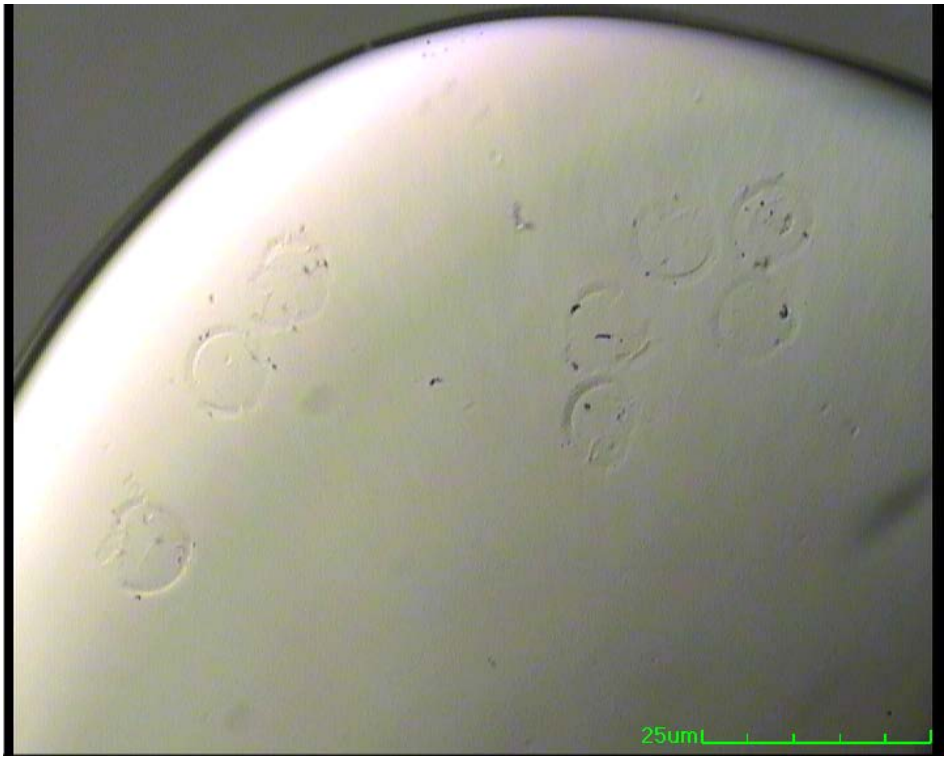

Figure 7.2.2 Figure demonstrates 8 zona pellucida halves before being placed into wash drops with sperm

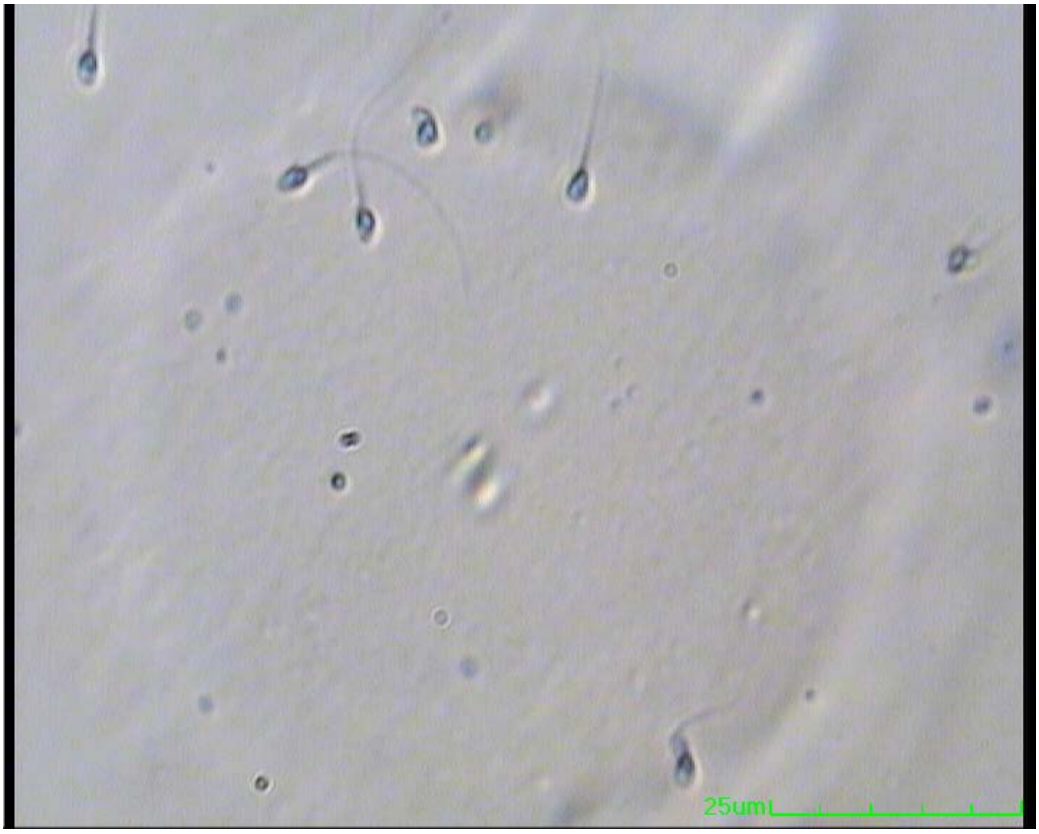

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Figure 7.2.3 Figure demonstrates magnified observation of sperm binding to zona pellucida.

Figure 7.2.4 Figure demonstrates Hemi-Zona Assay

7.2.3 Results

The results of sperm-zona pellucida binding with and without Rolipram are demonstrated in table 7.1. Figures presented represent the actual number of sperm tightly bound to each zona pellucida half. Visual examples from two oocytes are also demonstrated below (Figures 7.2.5 and 7.2.6).

Oocyte Number	Control or Treated half of zona pellucida	Number of sperm bound to zona pellucida
1	Control Rolipram	18 28
2	Control Rolipram	20 27
3	Control Rolipram	3 2
4	Control Rolipram	5 7
5	Control Rolipram	1 0
6	Control Rolipram	0 3
7	Control Rolipram	2 4
8	Control Rolipram	4 2
9	Control Rolipram	8 10
10	Control Rolipram	22 28
11	Control Rolipram	7 5
12	Control Rolipram	13 16

Table 7.1 Demonstrating the number of sperm bound to each human zona pellucida. Sperm incubated with or without Rolipram.

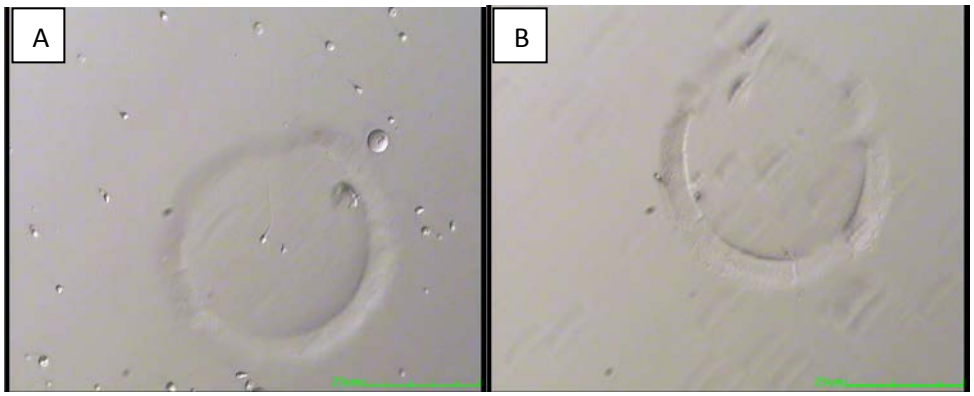

Figure 7.2.5 Demonstrating results from oocyte number 5 (see Table 1) a zona pellucida half with one sperm bound to the control (A) and no sperm bound to the treated half (B).

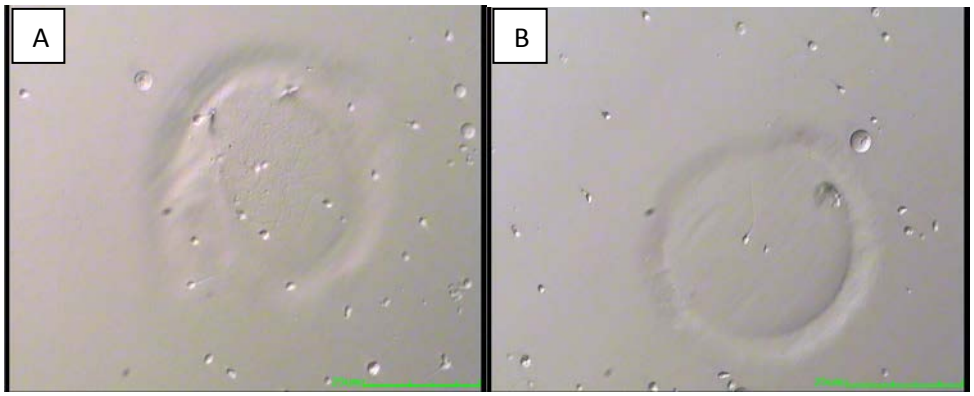

Figure 7.2.6 Demonstrating results from oocyte number 8 (see Table 7.1) a zona pellucida half with four sperm bound to the control (A) and two sperm bound to the treated half (B).

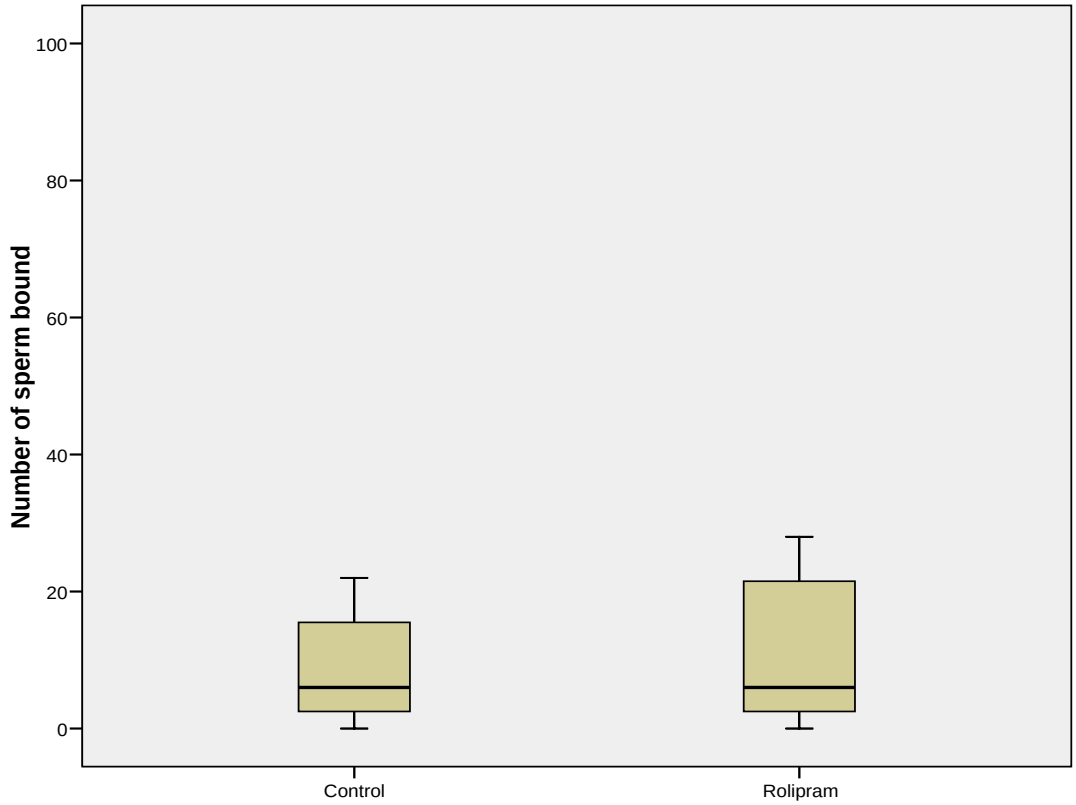

Figure 7.2.7 Graph demonstrates the mean number of sperm bound in to the zona pellucida of control and Rolipram treated groups.

There was a significant difference ($P<0.05$) in the number of sperm bound to the zona pellucida between the 80% fraction of ICSI sperm samples with and without the addition of Rolipram (mean 8.58 and 11.0 respectively).

7.2.4 Discussion

Sperm-zona pellucida binding is a well established technique used to predict the fertilisation potential and pregnancy outcome within infertile couples (Arslan et al.,

2006). This technique which has been carried out since the late 80s has been applied clinically to determine the fertilising competence of human sperm. The results above demonstrate a significant improvement in the number of sperm bound to human zona pellucida with the use of Rolipram.

One of the reasons patients undergo ICSI treatment is due to previous IVF fertilisation. Sperm may be incapable of binding to the zona pellucida and fusing with the oocyte due to poor motility and abnormal morphology. Embryologists have been able to overcome this barrier since ICSI was introduced in 1992. Whenever there is clinical doubt on the quality of a sperm sample, it is usually converted to ICSI, costing more money for the patient (if self-funding), more time consumed for the procedure and an increased risk of damaging the oocyte. If there are substances which provide known improvement in sperm quality, enhancing the fertilising potential of sperm, this quick judgement may not always be the case.

The molecular basis for which sperm binds to the zona pellucida is still an unresolved issue as the complete binding process has not yet been explained. Carbohydrate sequences on the zona pellucida have recently been determined (Pang et al., 2011) and proteins located on the sperm surface relating to zona pellucida binding have also been found (Redgrove et al., 2011). Insights into the molecular mechanisms that govern sperm-oocyte interaction are becoming apparent and the technique of sperm-zona pellucida binding is becoming further implicated, particularly in predicting fertilisation outcome. However, due to the limitations of access to ART laboratory equipment, knowledge of techniques and the ability to

research, this technique is rarely carried out for research purposes. Sperm-zona binding has never been investigated with regards to PDE inhibitors and their effects. This is a novel approach to investigate the effect of PDE inhibitors on sperm function. The results gained in this section suggest that sperm are more capable of binding to the zona pellucida after incubation with Rolipram. Since the sperm-zona binding technique is a well known predictor of fertilisation it may be suggested that with an increase in the number of sperm bound to the zona pellucida after incubation with Rolipram, an increase in fertilisation rate may occur. The clinical value of the addition of PDE inhibitors could add new tests to assess a couple's potential for fertilisation (e.g. semen analysis) or may in fact be used as a clinical step in sperm preparation with the view to improving success rates.

Chapter 8: General Discussion

The results in this study demonstrate a number of facts about the effect of PDE inhibitors on human sperm motility and function.

Incubating sperm with PDE inhibitors clearly increases the motility of samples from sub-fertile men attending an assisted conception unit for ICSI, predominantly the male factor infertility procedure. The clinical value of this is highly significant considering almost half of all ART procedures are of male factor infertility. The main element to male sperm inefficiency is poor motility and if enhanced, this could provide great potential for the couple and the clinic. An increase in sperm motility suggests an improvement. This may include the fertilisation potential for the couple and the alternative of a less time consuming procedure for the embryologist. In the past, it has been reported that PDE inhibitors do not have a positive effect of sperm motility. These studies however, have used samples collected from healthy sperm donors whose sperm, as shown in this study, cannot be improved as the number of motile cells is already high. The topic of PDE inhibitors and human sperm is still debatable and requires further investigation; however the basis for the evidence of the improvement of sperm samples with these inhibitors is already there.

This study then examined the potential of PDE inhibitors having a negative effect on sperm physiology. Although the importance of the stage of the acrosome reaction during sperm-oocyte interaction is to date clearly controversial, it was established that in this case induction of the acrosome reaction by PDE inhibitors would be a

negative effect. The acrosome of sperm incubated with PDE inhibitors remained intact in this study. It is unknown as to whether this is a beneficial effect as although PDE inhibitors do not seem to induce it, the acrosome reaction is a crucial step in the process of fertilisation and it is not known whether incubation with PDE inhibitors may in fact prevent it. Further studies are required to investigate the exact effect of PDE inhibitors on the state of sperm acrosome. Further to this, sperm DNA was examined to determine whether any damage is caused when sperm are incubated with PDE inhibitors. It would seem as though PDE inhibitors do not induce any damage to sperm cell DNA. Tests such as this are crucial to establish the clinical benefits of using PDE inhibitors during ART procedures. Besides the benefits PDE inhibitors have on sperm motility, to learn that they have a negative effect on sperm DNA would rule them out from clinical use completely. The results in this study demonstrate no negative effects of the drugs on sperm DNA. However, the TUNEL assay is a controversial technique as it is not a reliable test. More investigation would be required to determine any negative effects PDE inhibitors have on human sperm.

In this study sperm were then incubated with PDE inhibitors which have limited or no previous information. This was a novel approach to investigate the effect of PDE inhibitors on sperm motility as the previously used inhibitors (8-MeOM-IBMX and Rolipram) have already been studied. Milrinone, BRL and Papaverine which have not been extensively studied previously showed similar effects on sperm motility. These are encouraging results for future investigation. Non-specific inhibitors such as PTX have been extensively studied and it is well known that they have positive effects on sperm motility. Table 8.1 demonstrates some of the studies which have investigated this inhibitor at a clinical level giving the fertilisation and pregnancy

rates with the use of PTX. The significance values are in comparison to those without the PDE inhibitor. In comparison to PTX, very little is known of the effect of other PDE inhibitors on human sperm motility and function. The results in this thesis however are encouraging and provide an incentive to advance with the investigation of PDE inhibitors on human sperm motility.

Further from this, human sperm function was investigated to determine if PDE inhibitors not only give a positive effect on sperm motility, but sperm function in relation to the steps leading to fertilisation. The sperm penetration test was used to establish whether sperm are more able to swim through viscous media when incubated with PDE inhibitors. This mimics the journey of sperm to the oocyte through the female reproductive tract, an important step leading up to sperm-oocyte interaction. The results demonstrate there is a significant increase in the number of sperm at a marked distance when sperm have been incubated with PDE inhibitors. Reasons for this may include the fact that sperm swim faster when incubated with PDE inhibitors and are therefore more capable of dealing with viscosity than those without. These results show a positive relationship between PDE inhibitors and human sperm function. However, although more sperm may be able to reach a required distance, and possibly the oocyte in vivo, this does not necessarily mean PDE inhibitors aid sperm-oocyte interaction, the most important step in fertilisation.

A novel approach was taken to investigate the effect of PDE inhibitors on the ability of sperm to bind to human oocyte zona pellucida. Sperm-zona pellucida binding has previously been used as a way of predicting fertilisation. This is a well known technique which has mainly been carried out using cadaver or animal oocytes.

Studies have tested this technique on human oocytes including this thesis. However, not many studies have carried out sperm-zona pellucida binding using resources from an assisted conception unit, techniques so closely applied to daily clinical work and patients attending the clinic for treatment. It is important to mention these are modern resources and techniques which would not have been available when these previous studies were carried out. Further to this, no studies have examined the effect of PDE inhibitors on the ability of sperm to bind to human oocyte zona-pellucida in this situation. The results in this thesis suggest that PDE inhibitors have a positive effect on the ability of sperm to bind to the zona pellucida since more sperm were found bound after incubation with a PDE inhibitor. These results are again encouraging and give reason to investigate the effects of PDE inhibitors on human fertilisation. There were many difficulties with this technique. One of the problems and probably the most important was the use of germinal vesicle and metaphase I oocytes. Since metaphase II oocytes are used in the ART procedures of patient treatment, this is the closest to mimicking the actual IVF procedure. Results gained may therefore not be truly accurate of what would occur naturally as it is known immature oocytes do not fertilise. This may be the reason for the observation of such low sperm numbers bound to the zona pellucida. Overall, this technique was as accurate as possible with the aim of gaining enough information to determine whether PDE inhibitors have a negative effect on the ability of sperm to bind to the zona pellucida. These preliminary results demonstrate that sperm still bind in the presence of the inhibitor.

It is unlikely going by the results gained that PDE inhibitors cause a negative effect on the ability of sperm to bind to the zona pellucida, however further investigation is required in addition to these preliminary results to establish PDE inhibitors as clinically viable.

	Author (Year)	**Control Group (n=...)**	**Study Design**	**Concentration used**	**Infertility Treatment**	**Number Fertilised (%)**	**Number Pregnancies (%)**	**Abnormalities/ Miscarriages/ abortions**	**Significance**
PTX	(Yovich et al., 1988)	9	PC	1mg/ml	PROST	6 66.6 %	5 56%	1 miscarriage	P<0.05
	(Yovich et al., 1990)	57	PC	1mg/ml	PROST TEST	33.8 % 47.4 %	16 30%	2 blighted ovum pregnancies	P<0.001
	(Tasdemir et al., 1993)	11	PC	10µM	IVF	-	5 45.4%	Not mentioned	P<0.001
	(Tournaye et al., 1993)	22	PC	3.6mM	IVF	8 36%	2 9.1%	0	P<0.05
	Negri et al., 1996	40	PC	1mg/ml	IUI	-	11 27.5%	4 abortions	P<0.05
	(Stone et al., 1999)	932	RC	3.0mmol/L	IUI	-	159 17.1%	20 spontaneous abortions	P<0.001

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Appendix

Investigating the effects of phosphodiesterase inhibitors on human sperm motility and function

Repeated measures ANOVA was used to assess the effect of phosphodiesterase inhibitors over 0.5, 1 and 2 hour incubation periods and students paired t-test where readings were taken at 1 hour only. Statistical tests were carried out in SPSS version 19.

2.3.1 Effect of PDE inhibitors on 'healthy' donor population

8-MeOM-IBMX on 80% fraction donor

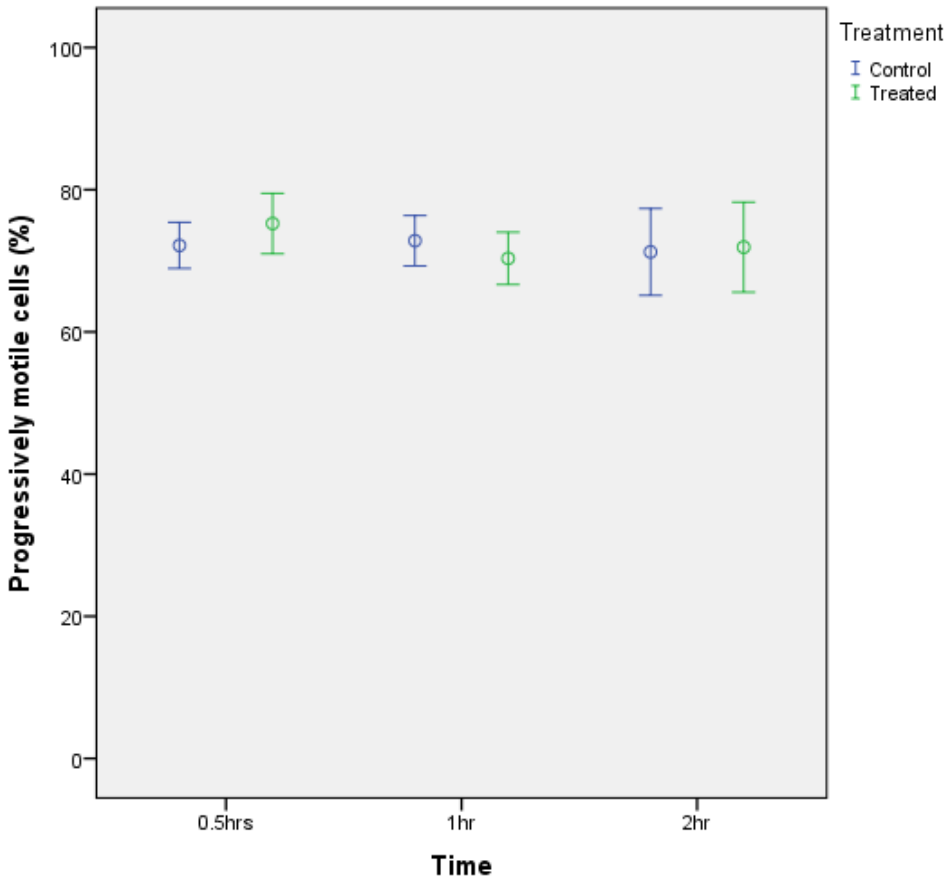

Figure 1. Graph demonstrates the mean percentage of progressively motile cells (±SEM) in the 80% fraction of donor samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P=0.798).

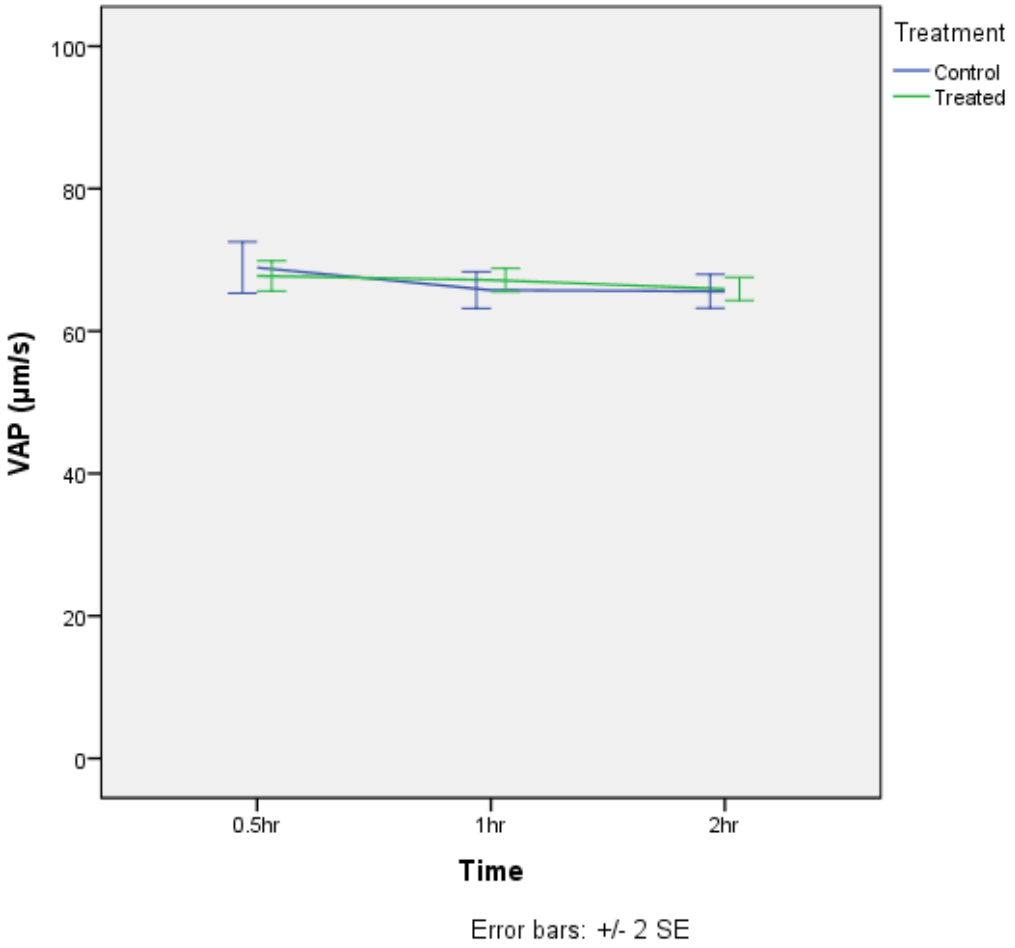

Figure 2. No differences could be found between VAP levels recorded over time (ANOVA P=0.530) for the 80% fraction of donor samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation.

Rolipram on 80% fraction donor

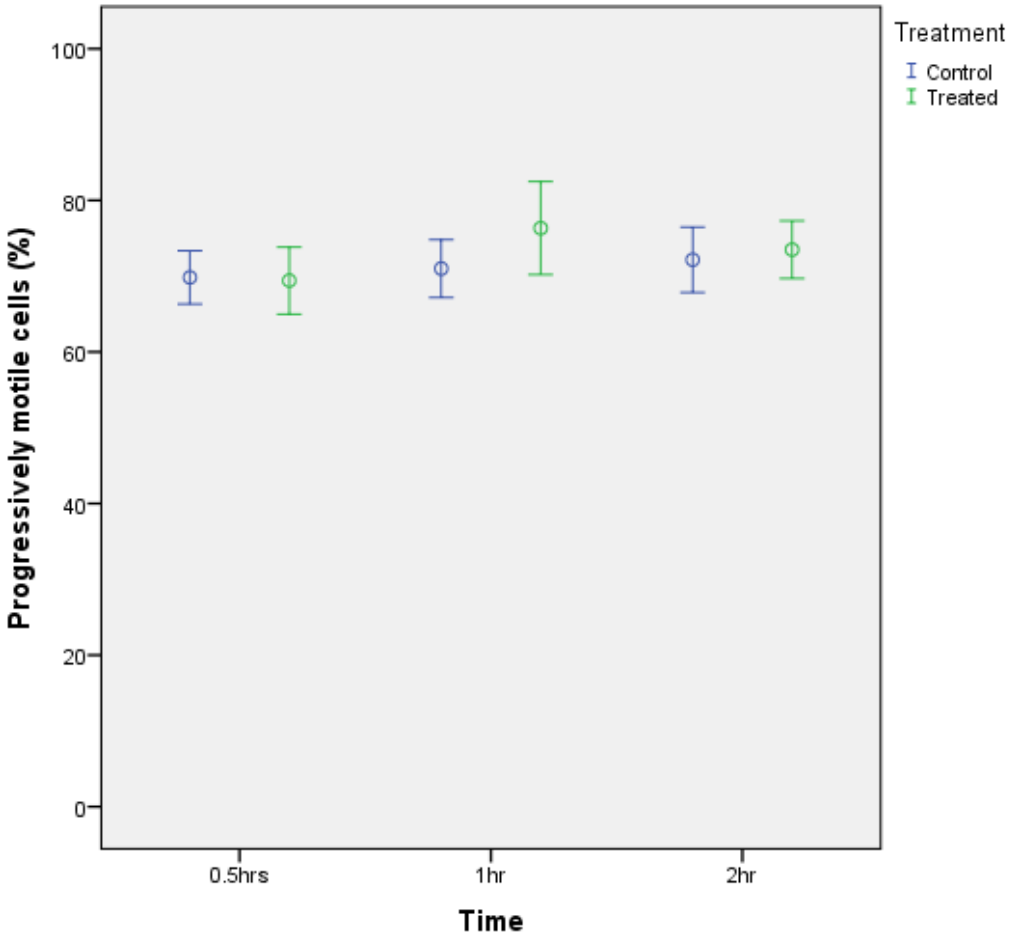

Figure 3. Graph demonstrates the mean percentage of progressively motile cells (±SEM) in the 80% fraction of donor samples with or without Rolipram after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P=0.697).

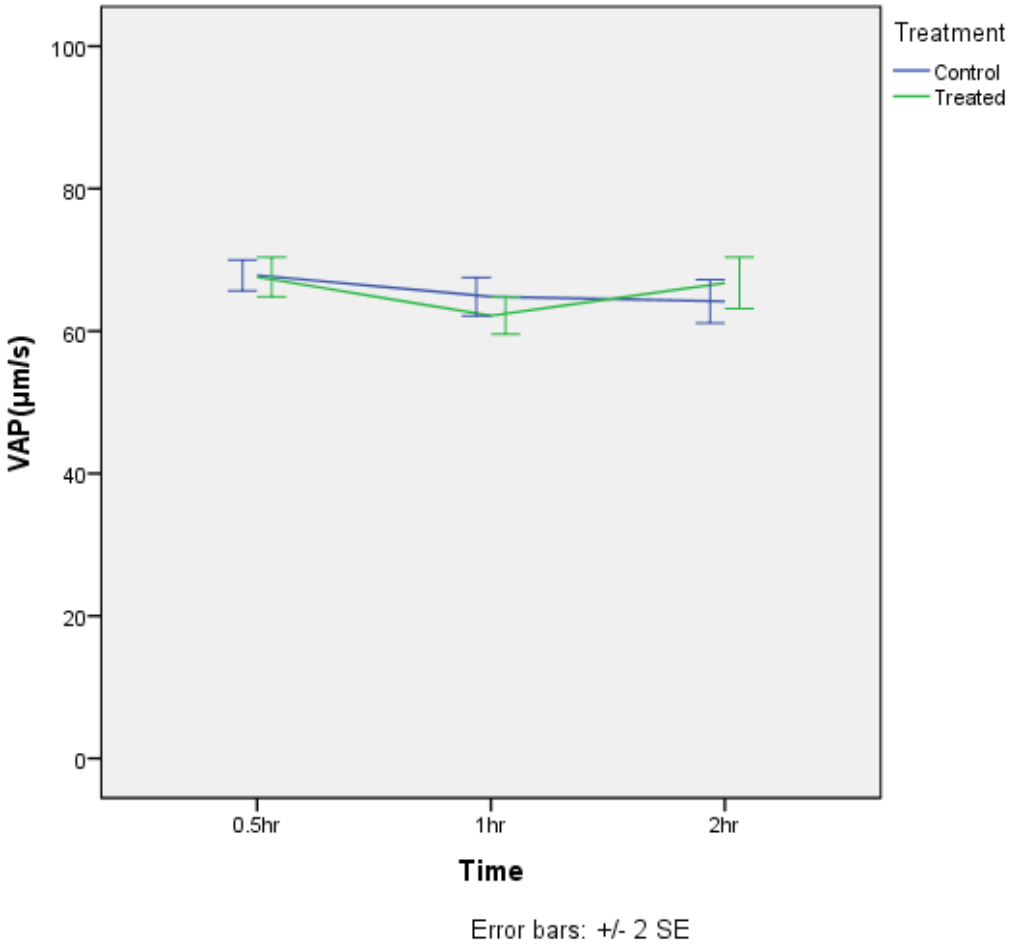

Figure 4. There was no statistical difference between VAP of control and treated over time (ANOVA P=0.911) for the 80% fraction of donor samples with or without Rolipram after 0.5, 1 and 2 hour incubation.

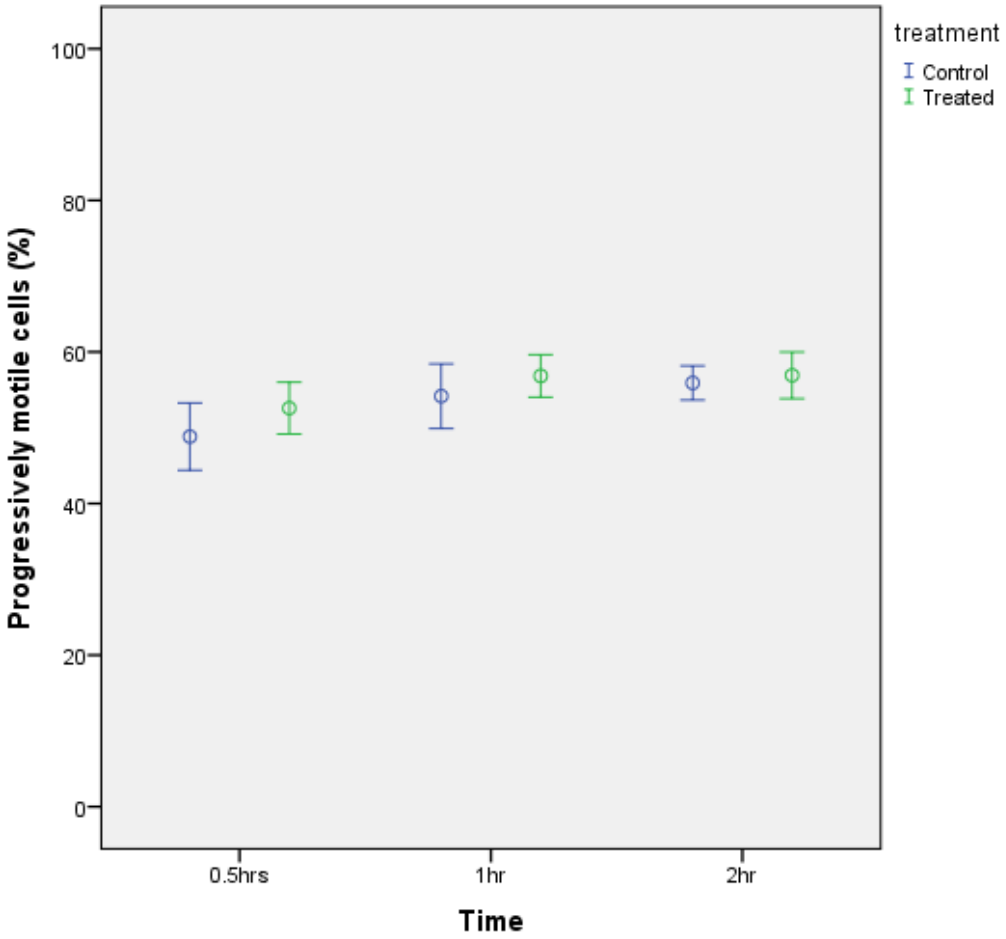

Figure 5. Graph demonstrates the mean percentage of progressively motile cells (±SEM) in the 40% fraction of donor samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

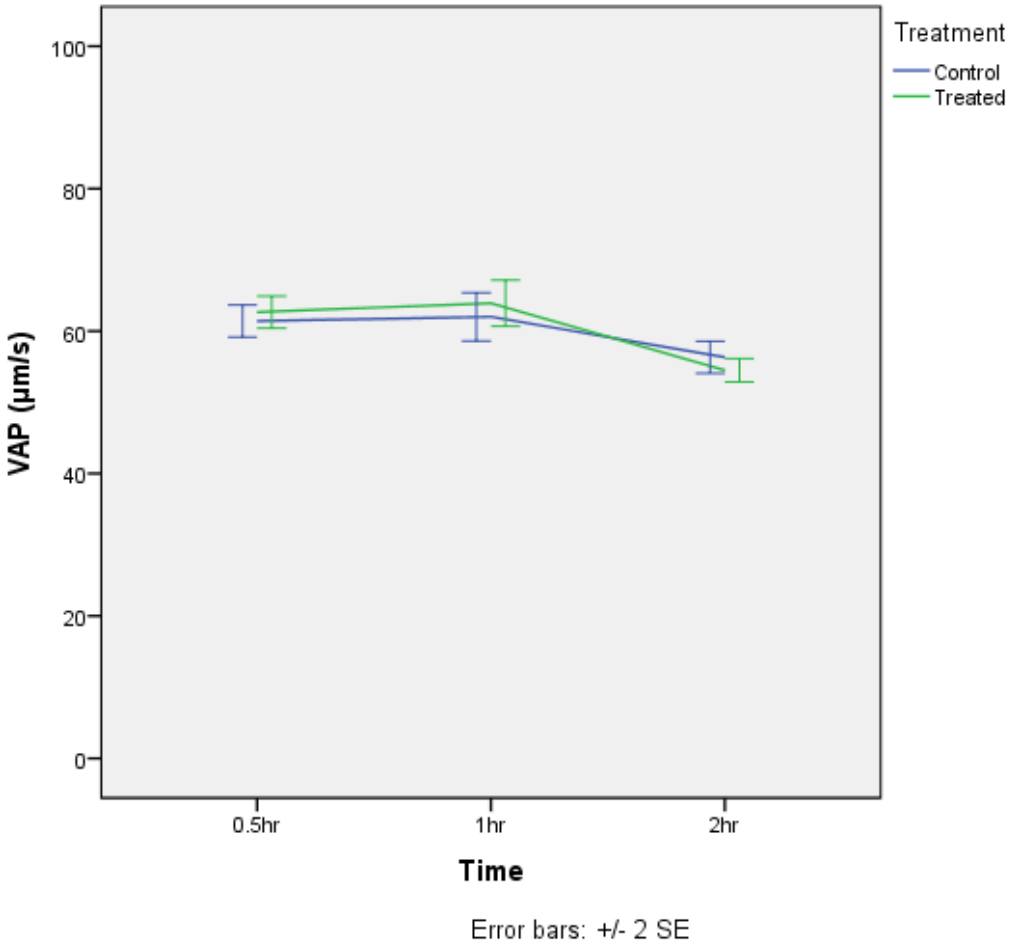

Figure 6. Graph demonstrates the effect of 8-MeOM-IBMX on the average path velocity of the 40% fraction of donor samples (n=3) over time. There is no statistically significant difference between control and treated over time (P=0.668).

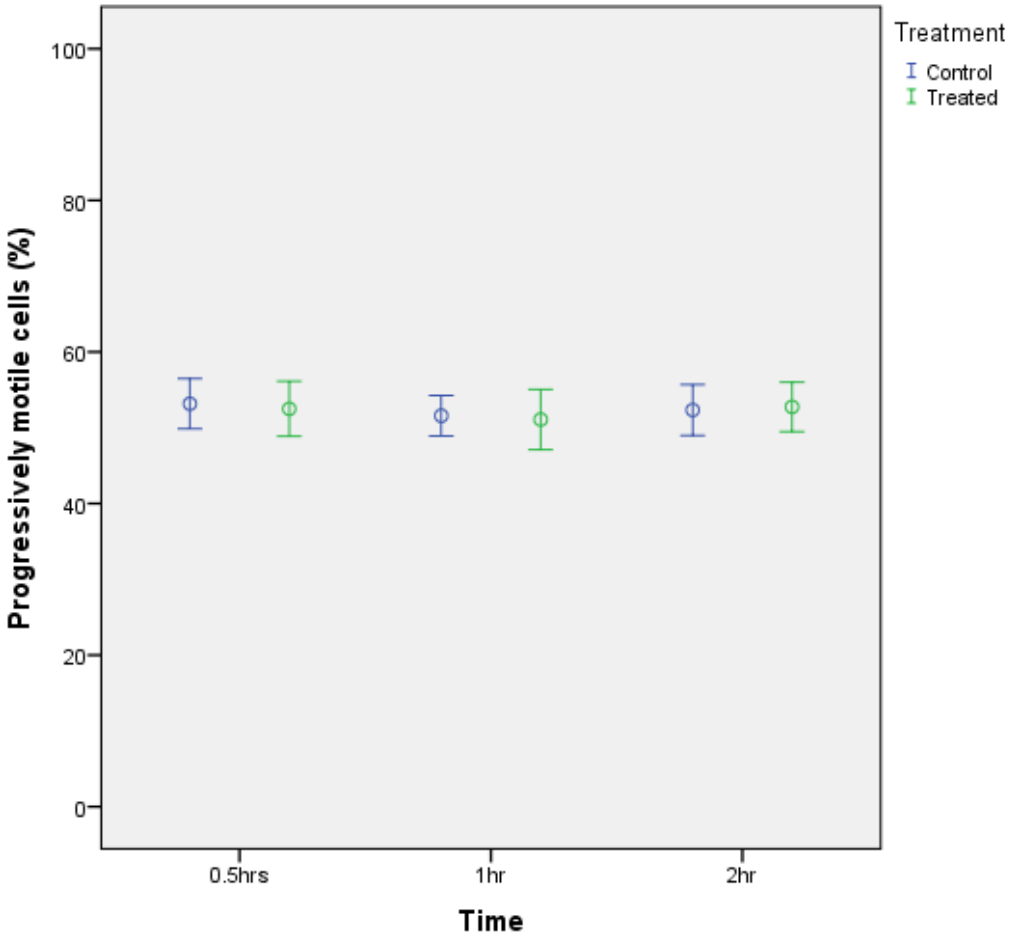

Figure 7. Graph demonstrates the mean percentage of progressively motile cells (±SEM) in the 40% fraction of donor samples with or without Rolipram after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

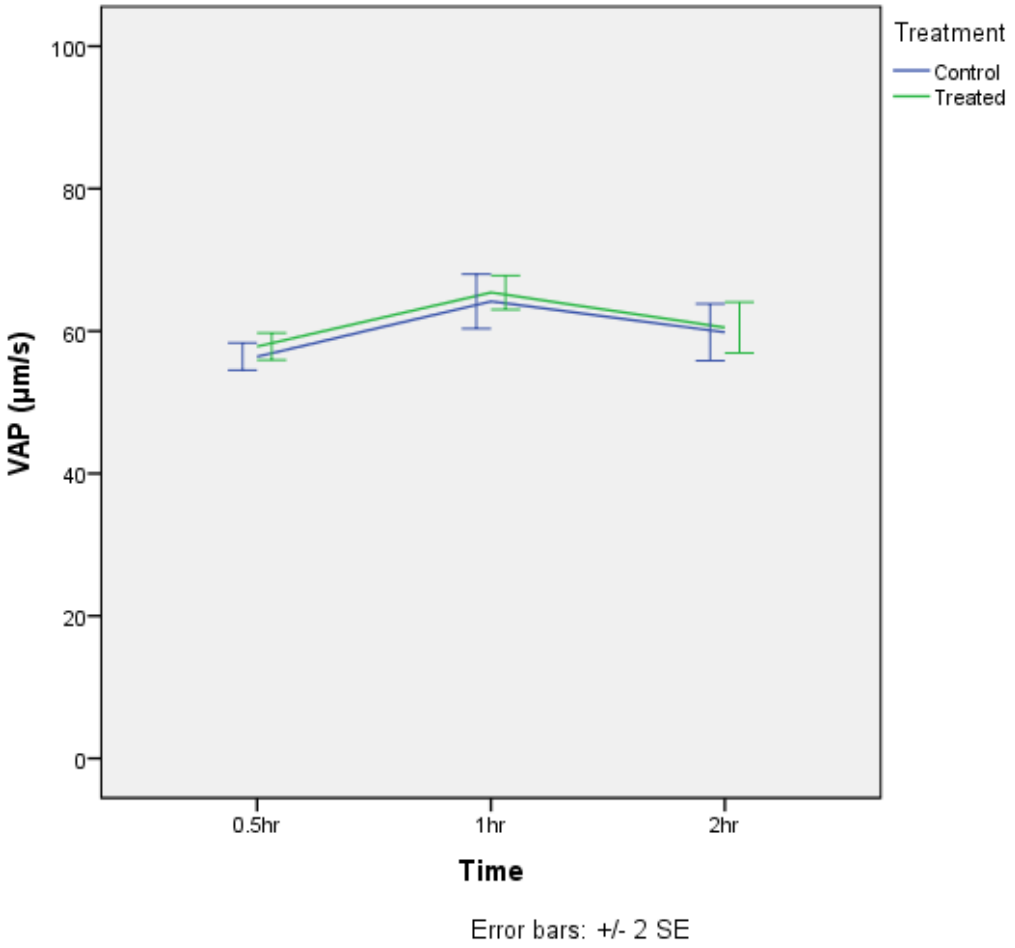

Figure 8. Graph demonstrates the effect of Rolipram on the average path velocity of the 40% fraction of donor samples (n=3) over time. There is no statistically significant difference between control and treated over time (P=0.347).

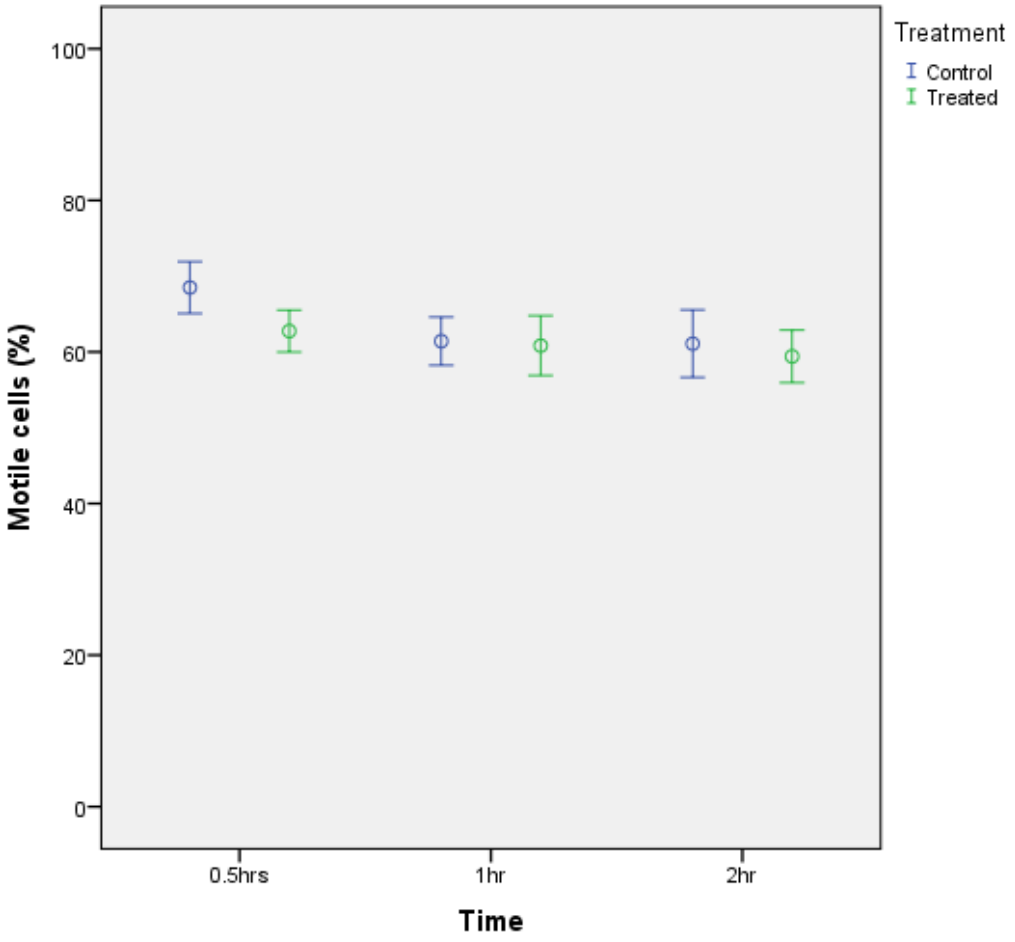

Figure 9. Graph demonstrates the mean percentage of motile cells in the 80% fraction of patient samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

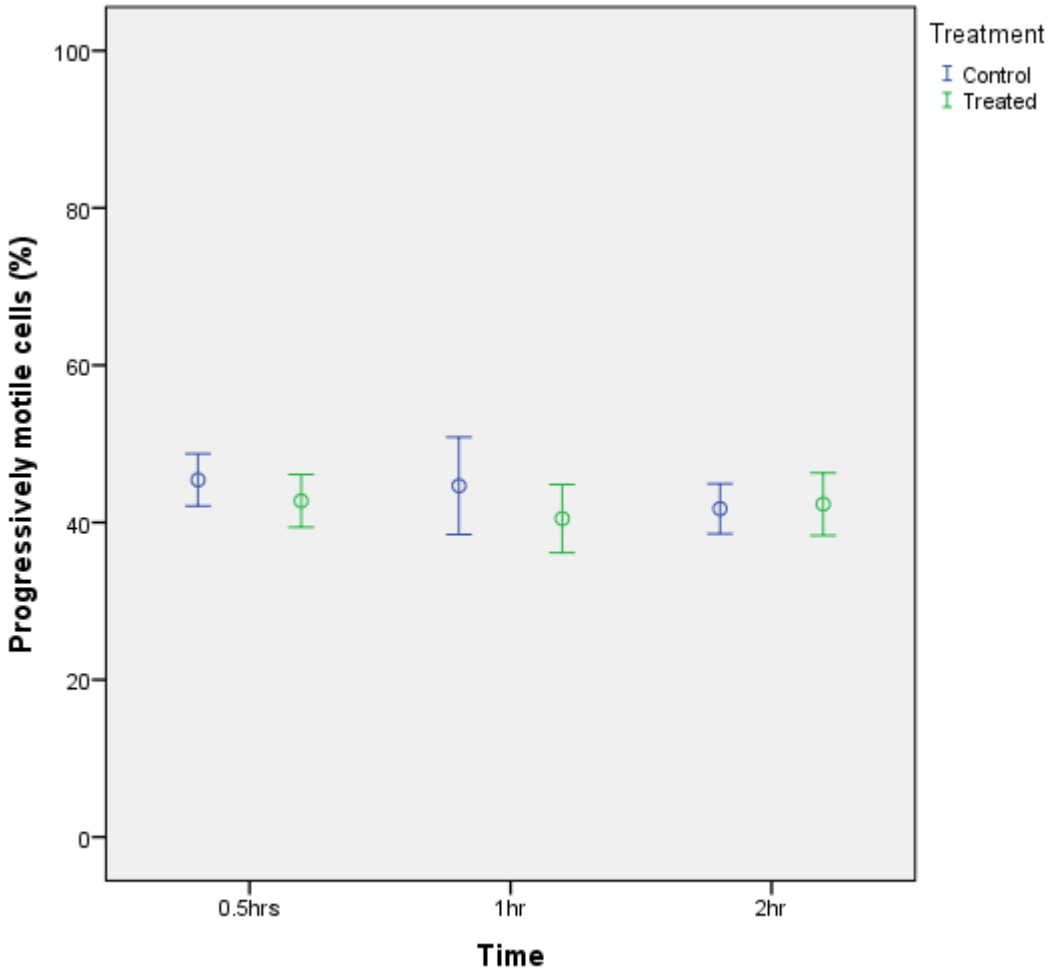

Figure 10. Graph demonstrates the mean percentage of progressively motile cells in the 80% fraction of patient samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

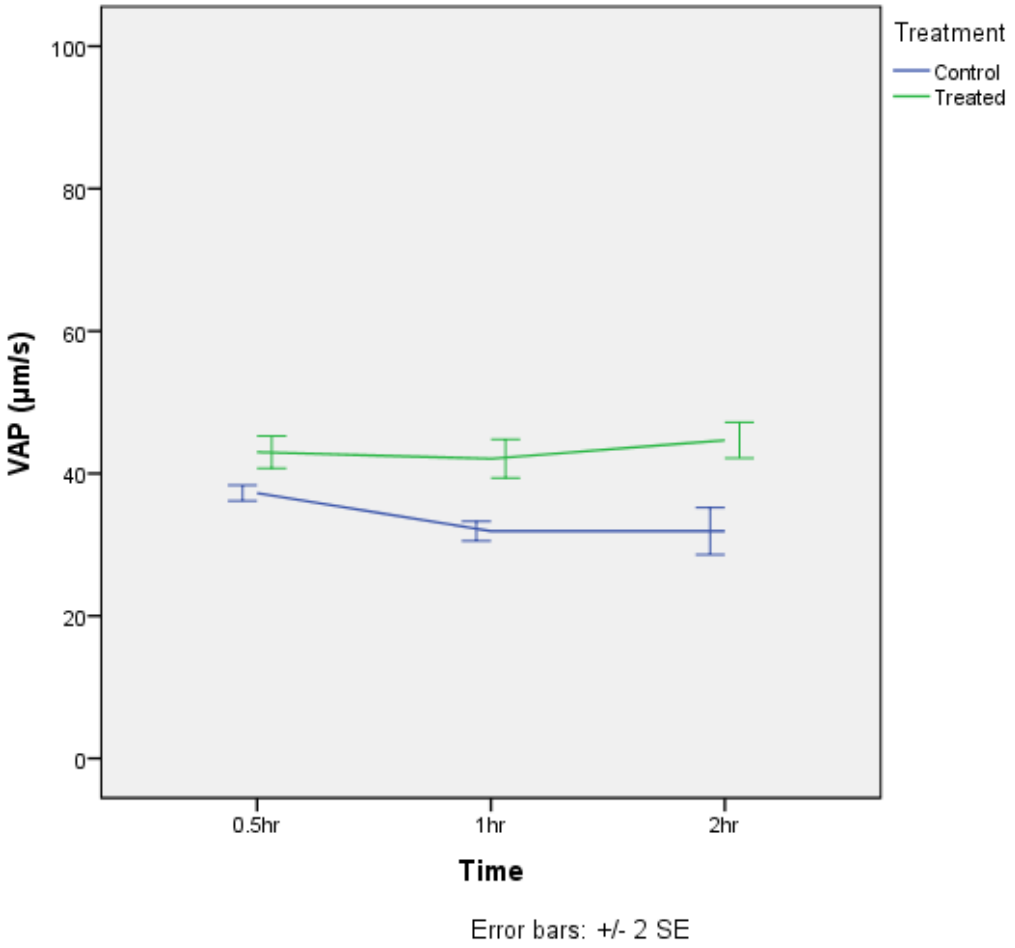

Figure 11. Graph demonstrates the effect of 8-MeOM-IBMX on the average path velocity of the 80% fraction of patient samples (n=3) over time. There is a statistically significant difference between control and treated over time (P<0.000).

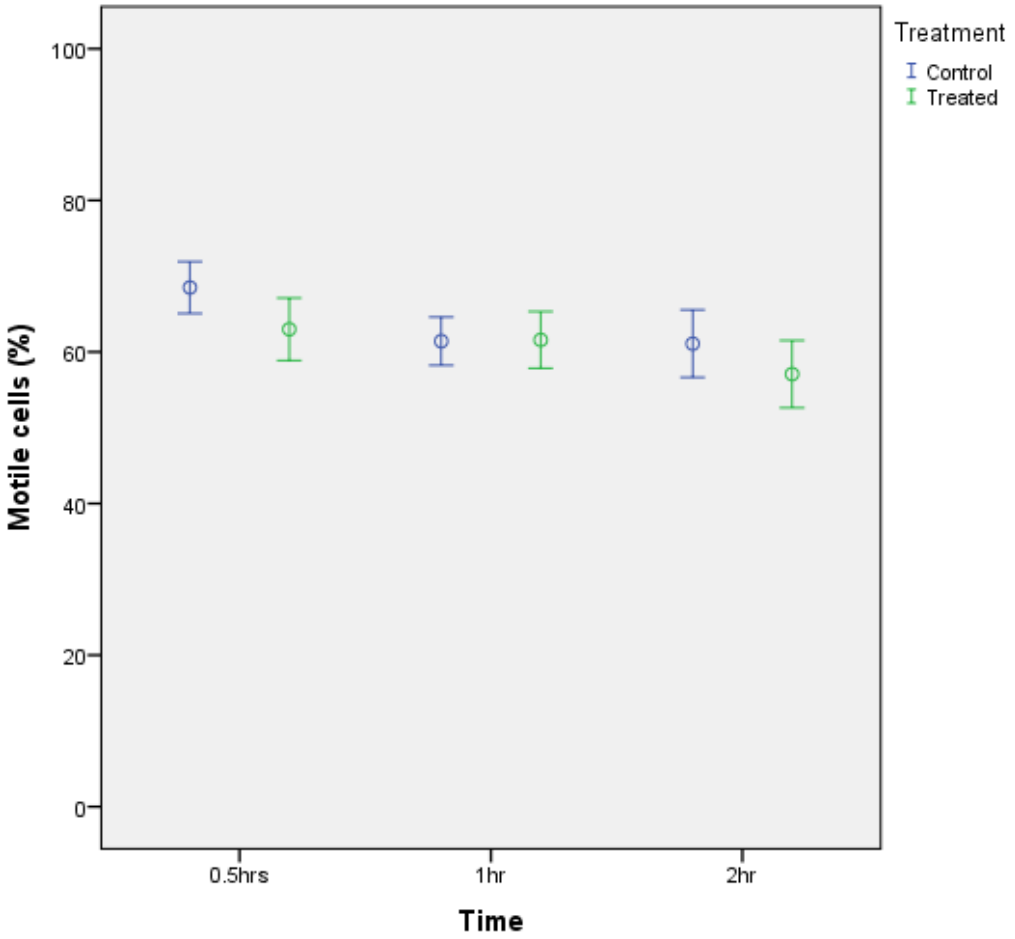

Figure 12. Graph demonstrates the mean percentage of motile cells in the 80% fraction of patient samples with or without Rolipram after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

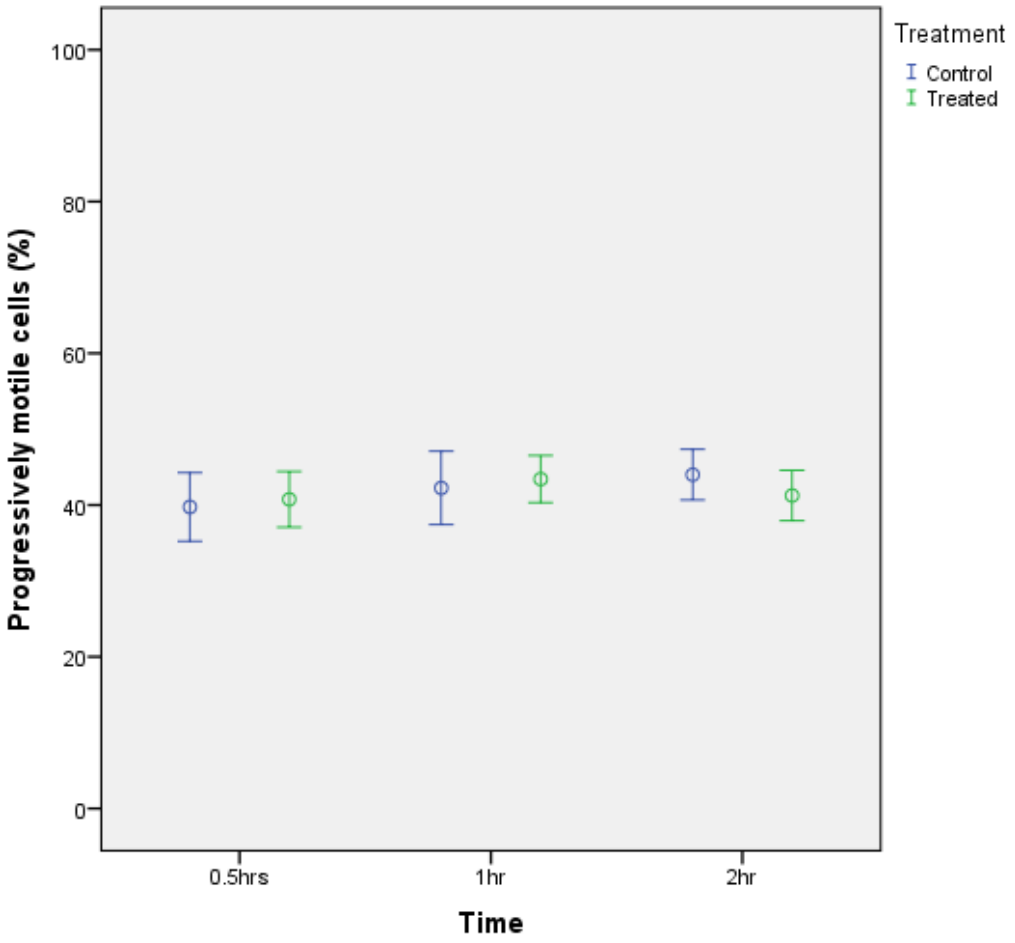

Figure 13. Graph demonstrates the mean percentage of progressively motile cells in the 80% fraction of patient samples with or without Rolipram after 0.5, 1 and 2 hour incubation. There was no statistically significant difference between control and treated at any time point (P>0.05).

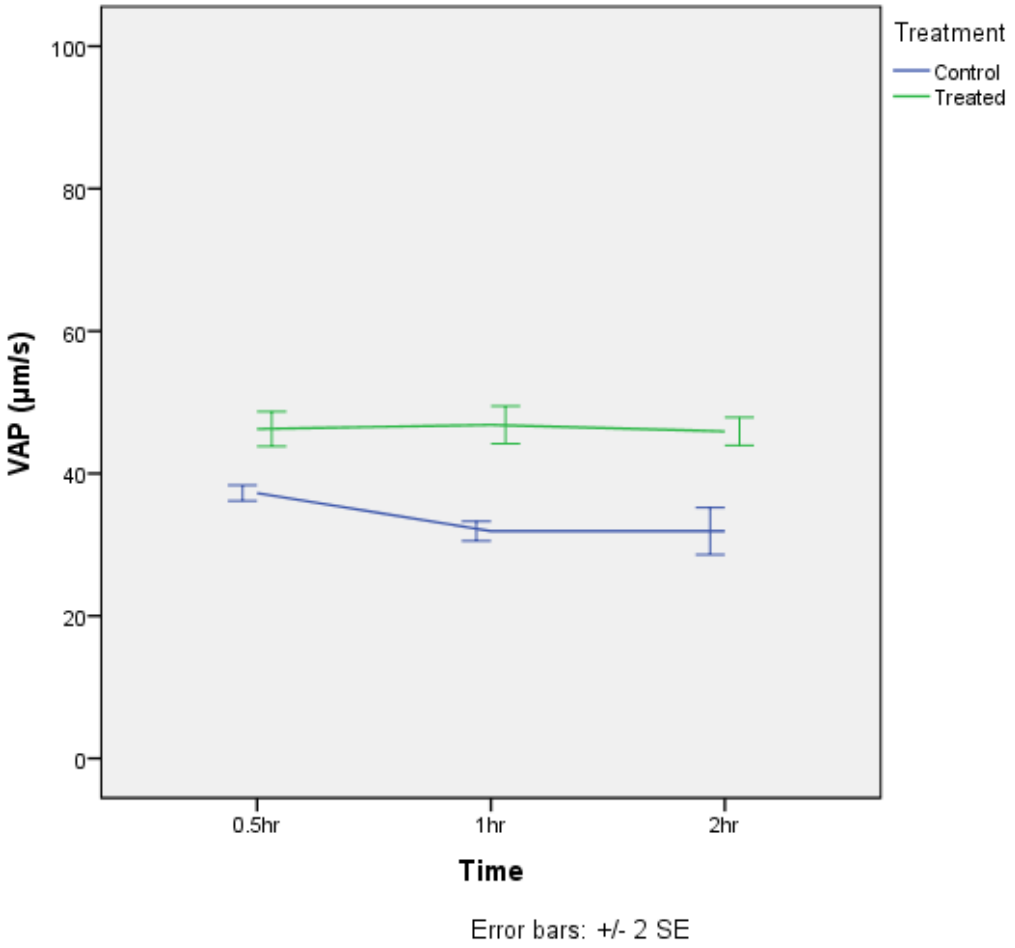

Figure 14. Graph demonstrates the effect of Rolipram on the average path velocity of the 80% fraction of patient samples (n=3) over time. There is a statistically significant difference between control and treated over time (P<0.000).

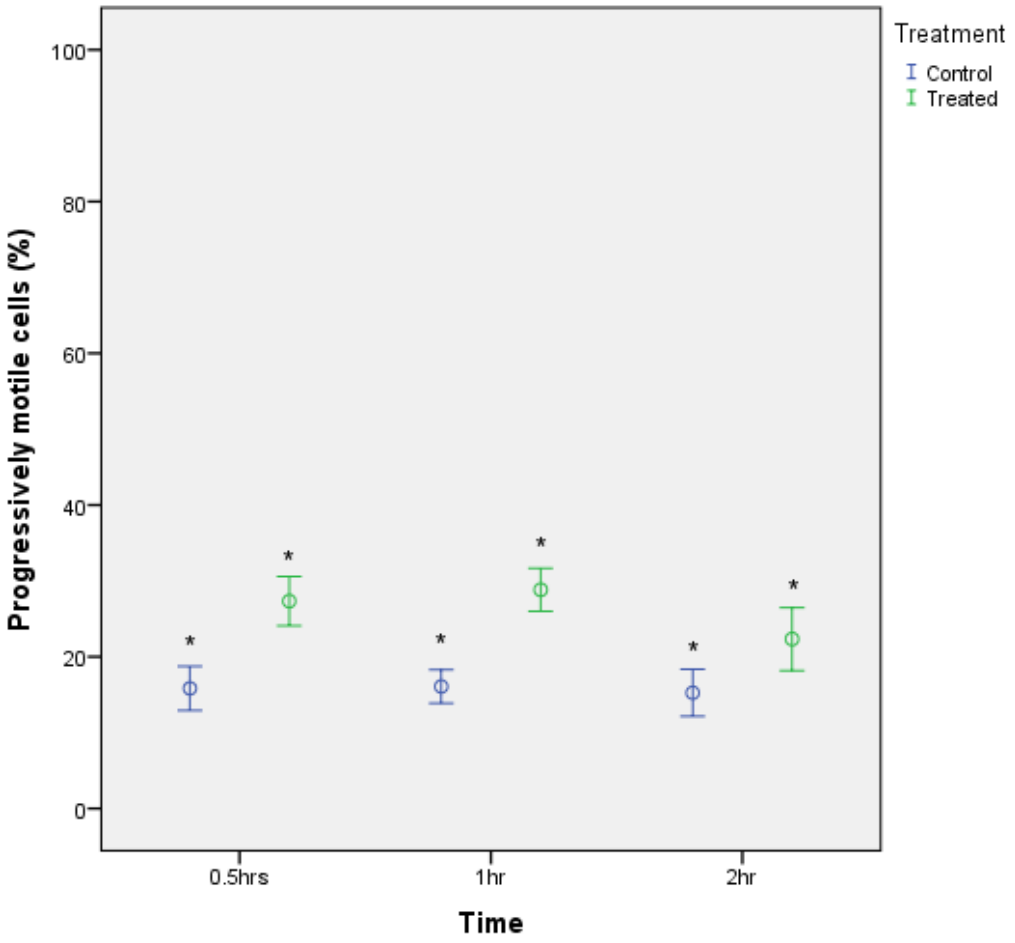

Figure 15. Graph demonstrates the mean percentage of progressively motile cells in the 40% fraction of patient samples with or without 8-MeOM-IBMX after 0.5, 1 and 2 hour incubation. There was a statistically significant difference between control and treated at each time point (P<0.001).

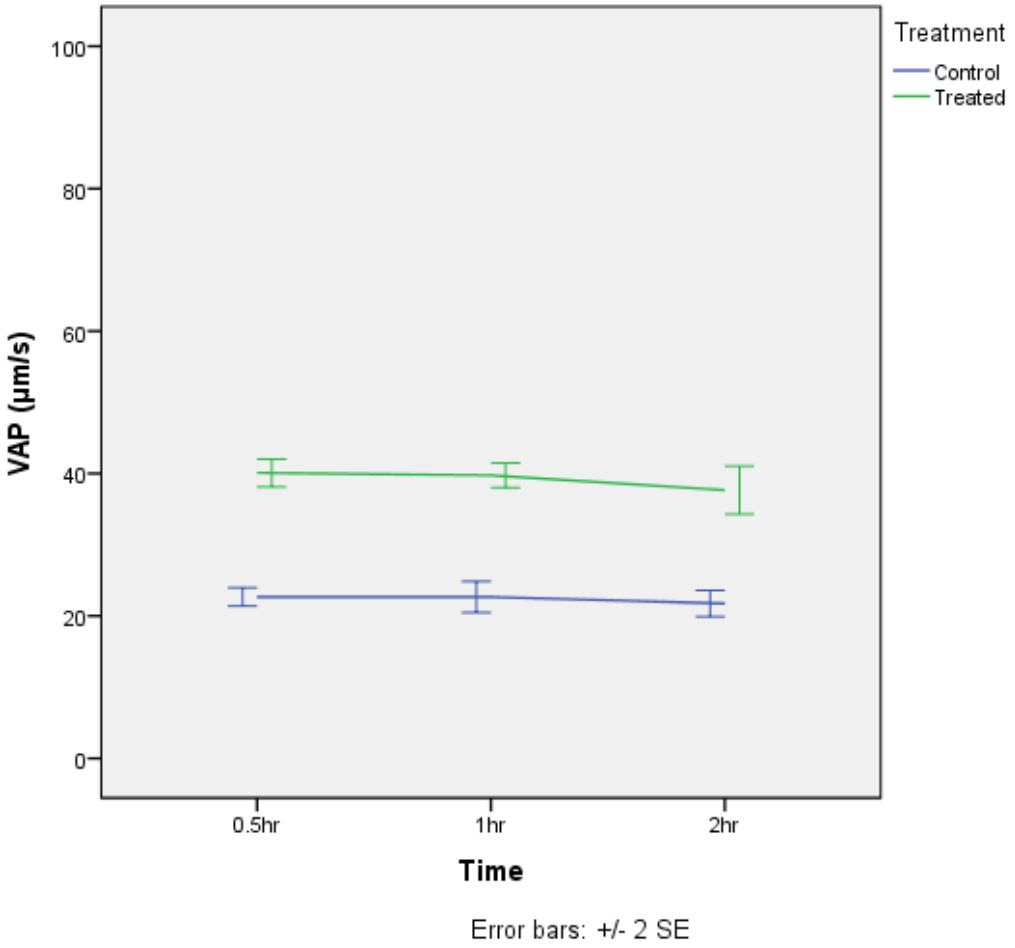

Figure 16. Graph demonstrates the effect of 8-MeOM-IBMX on the average path velocity of the 40% fraction of patient samples (n=3) over time. There is a statistically significant difference between control and treated over time (P<0.001).

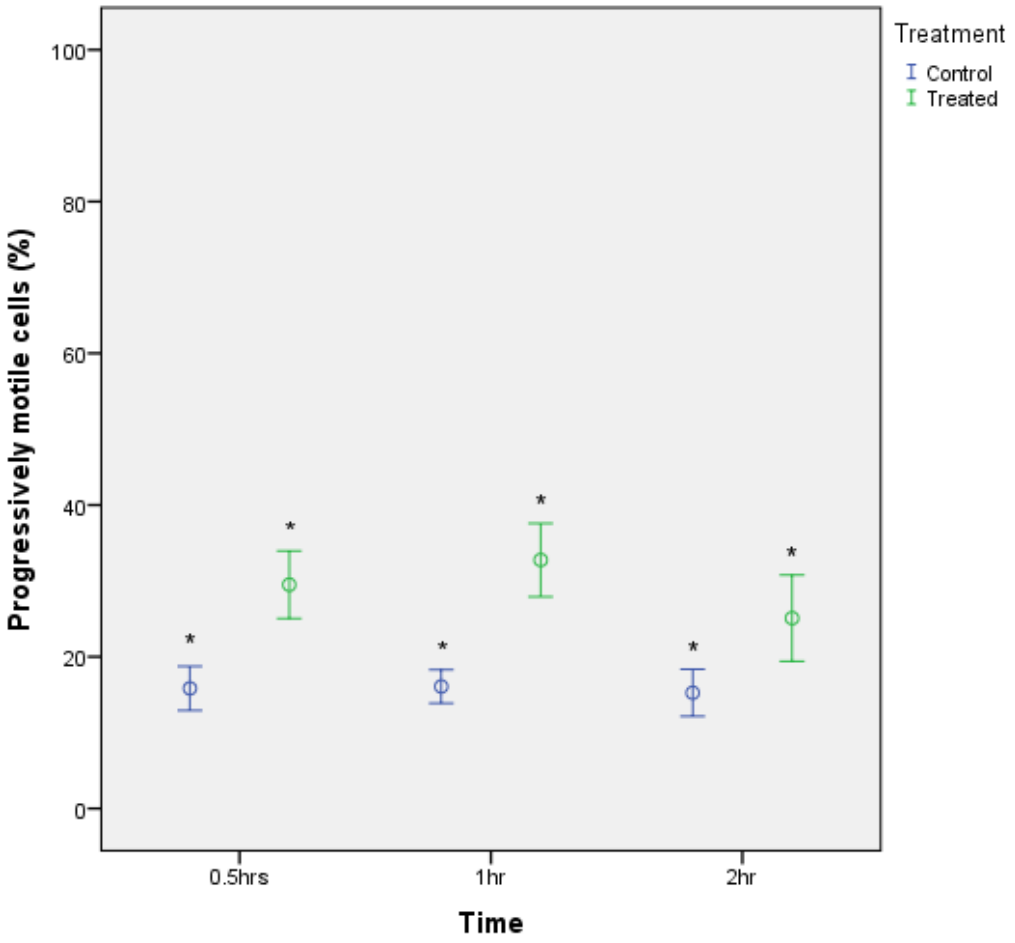

Figure 17. Graph demonstrates the mean percentage of progressively motile cells in the 40% fraction of patient samples with or without Rolipram after 0.5, 1 and 2 hour incubation. There was a statistically significant difference between control and treated at each time point (P<0.001).

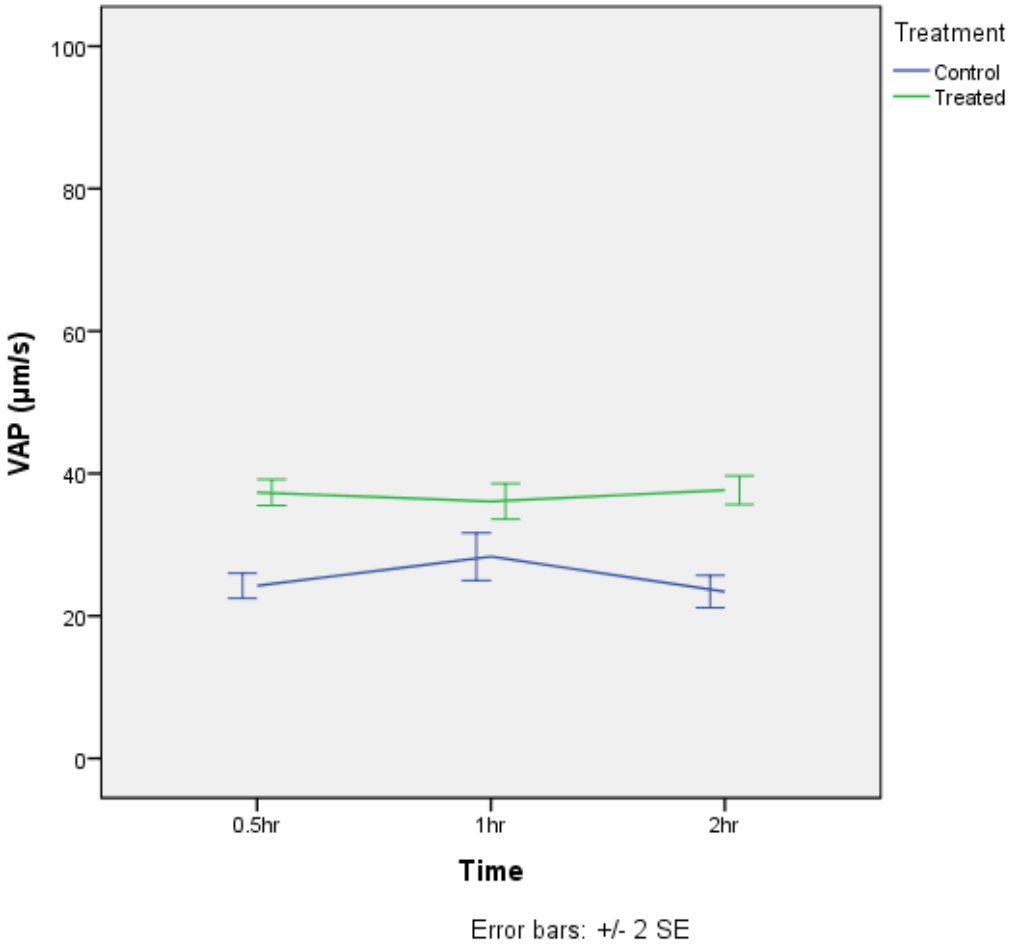

Figure 18. Graph demonstrates the effect of Rolipram on the average path velocity of the 40% fraction of patient samples (n=3) over time. There is a statistically significant difference between control and treated over time (P<0.001).P<0.001

Controls

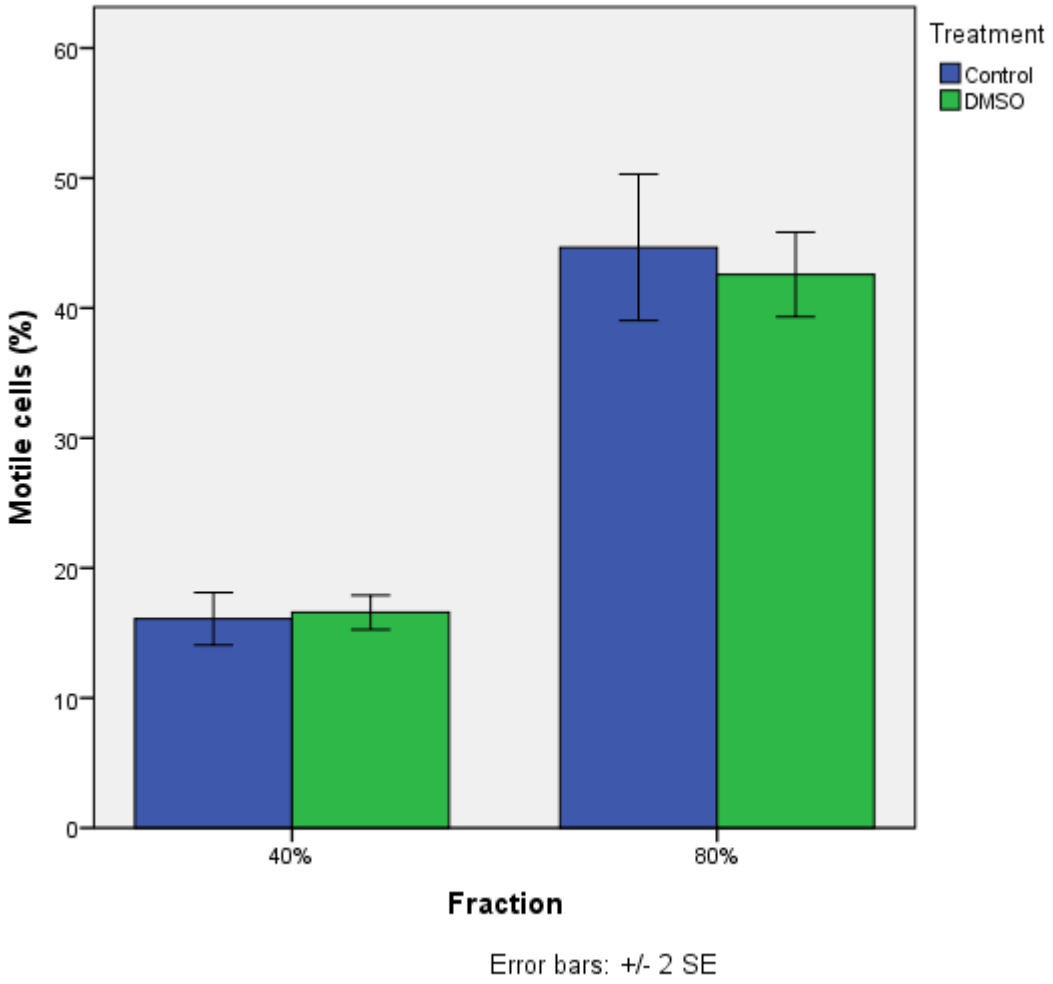

Figure 19. Graph demonstrates effect of DMSO on the mean percentage of motile cells in the 40% and 80% fraction of patient samples (n=3) with or without Rolipram after 1 hour incubation. There was no significant difference between control and treated in either the 40% or 80% fraction (P=0.713 and P=0.550 respectively).

Figures from presentation

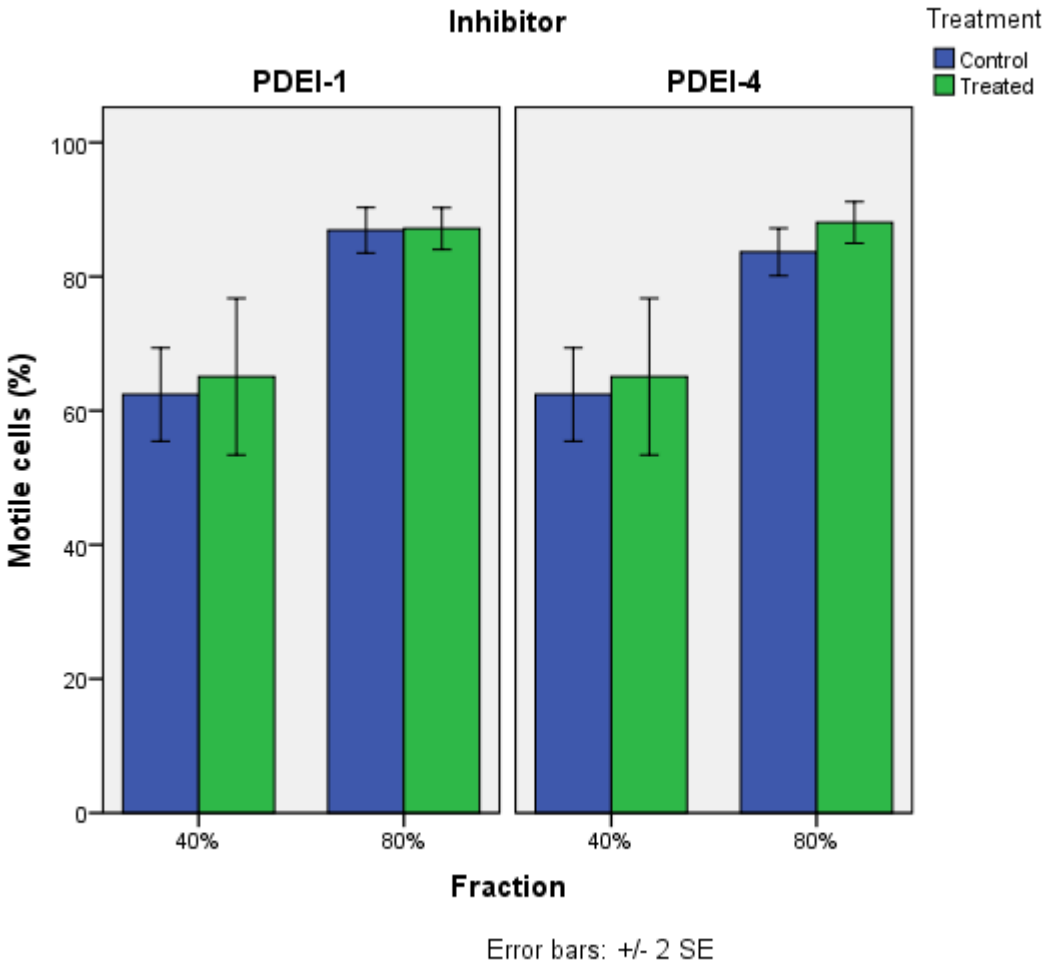

Figure 20. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the % of motile cells on the 40 and 80% fractions of donor samples (n=3). There is no significant difference between:

PDEI-1 40% (P=0.608)

PDEI-1 80% (P=0.893)

PDEI-4 40% (P=0.608)

PDEI-4 40% (P=0.098)

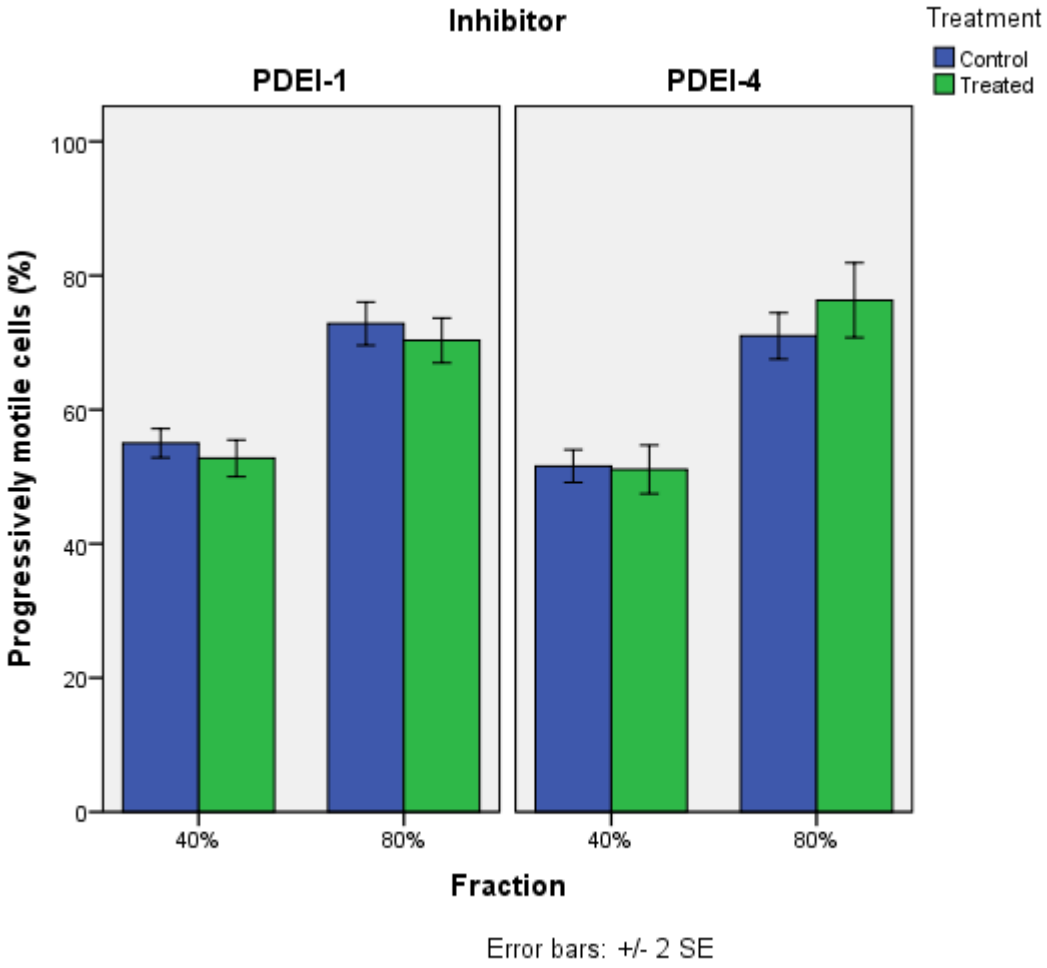

Figure 21. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the % of progressively motile cells in the 40 and 80% fractions of donor samples (n=3). There is no significant difference between:

PDEI-1 40% (P=0.295)

PDEI-1 80% (P=0.387)

PDEI-4 40% (P=0.818)

PDEI-4 40% (P=0.147)

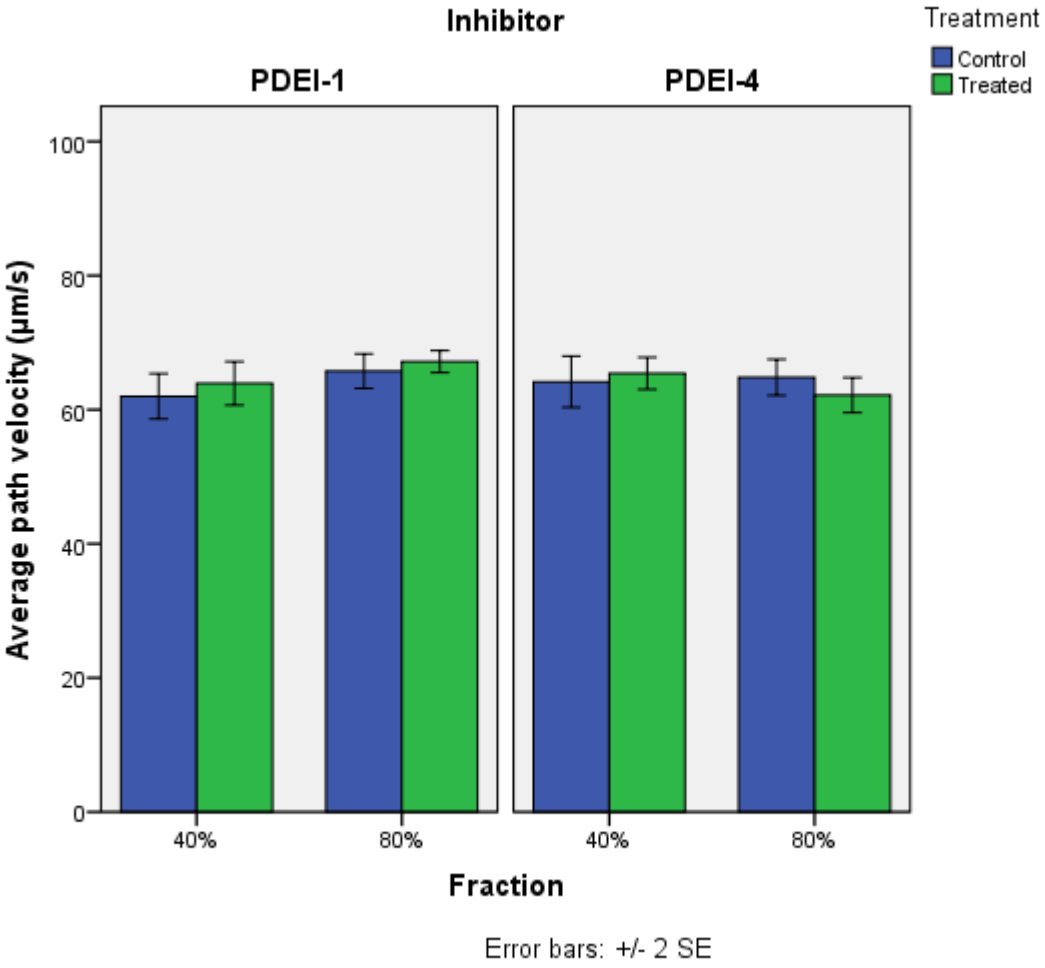

Figure 22. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the average path velocity in the 40 and 80% fractions of donor samples (n=3). There is no significant difference between:

PDEI-1 40% (P=0.452)

PDEI-1 80% (P=0.311)

PDEI-4 40% (P=0.566)

PDEI-4 40% (P=0.169)

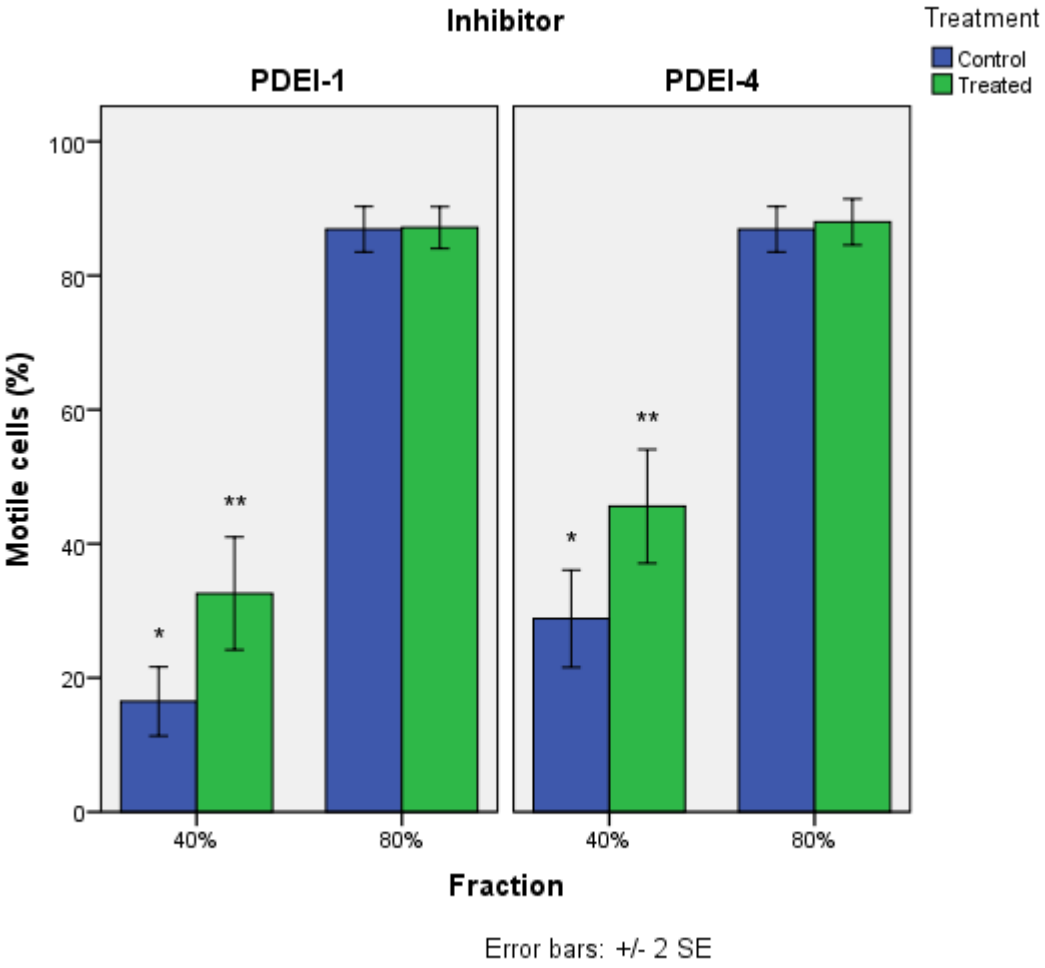

Figure 23. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the % motile cells in the 40 and 80% fractions of patient samples (n=3). There was a significant difference between the 40% fraction but not the 80%:

PDEI-1 40% (P<0.000)

PDEI-1 80% (P=0.893)

PDEI-4 40% (P=0.008)

PDEI-4 40% (P=0.418)

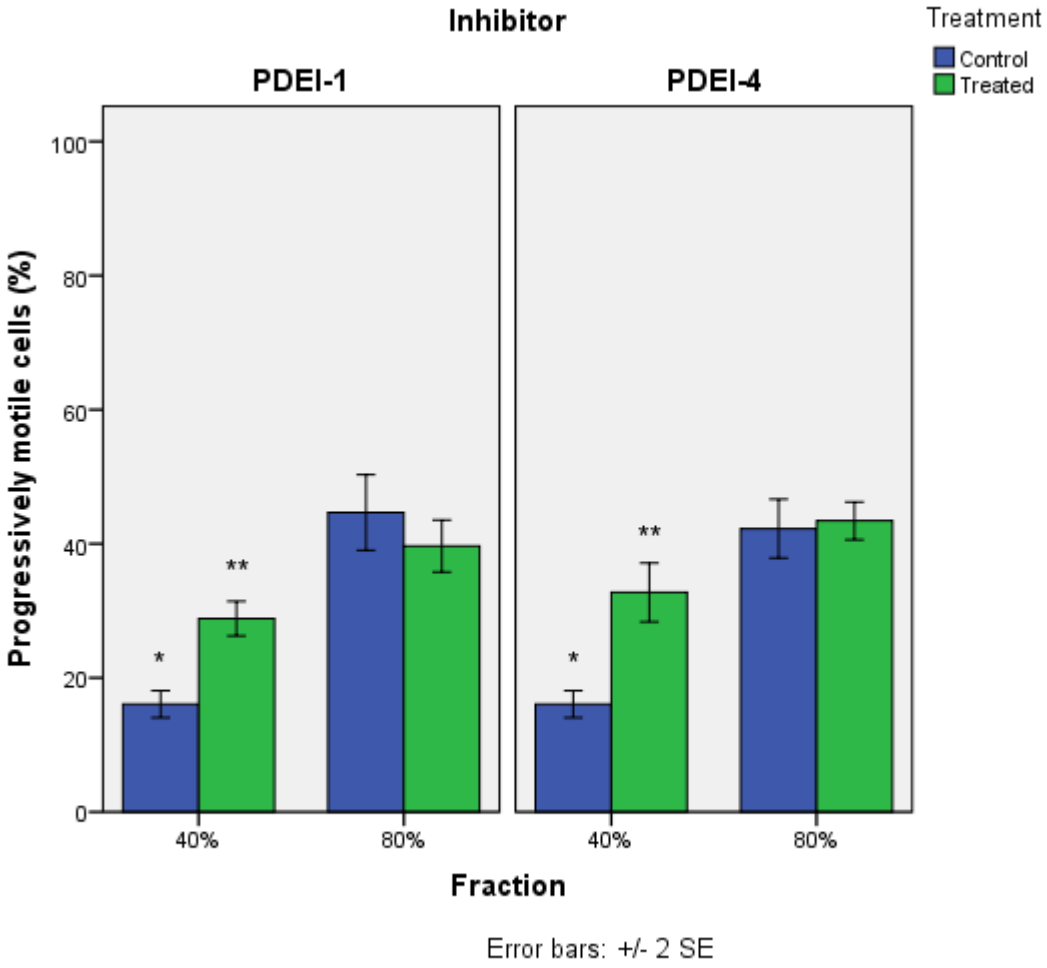

Figure 24. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the % progressively motile cells in the 40 and 80% fractions of patient samples (n=3). There was a significant difference between the 40% fraction but not the 80%:

PDEI-1 40% (P<0.000)

PDEI-1 80% (P=0.149)

PDEI-4 40% (P<0.000)

PDEI-4 40% (P=0.591)

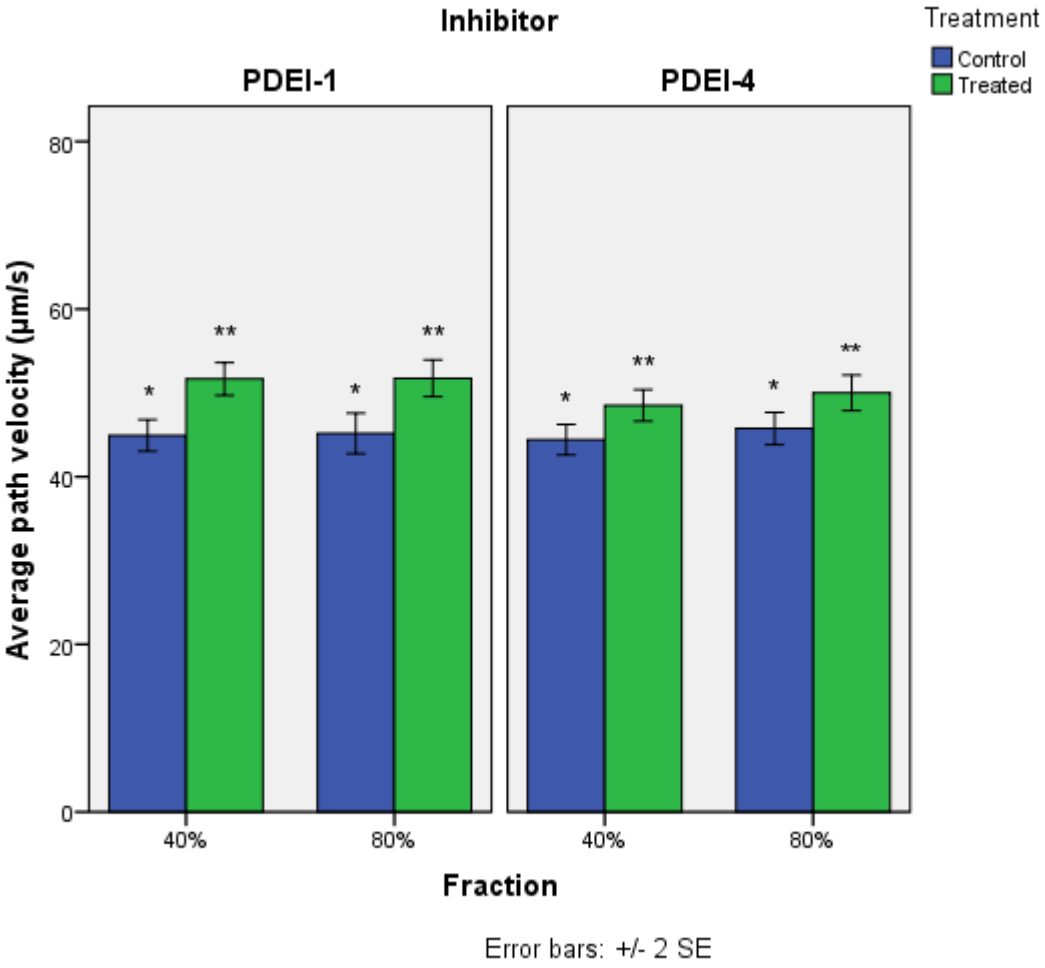

Figure 25. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX) and PDEI-4 (Rolipram) on the average path velocity in the 40 and 80% fractions of patient samples (n=3). There was a significant difference between control and treated in all fractions

PDEI-1 40% (P<0.000)

PDEI-1 80% (P=0.003)

PDEI-4 40% (P=0.009)

PDEI-4 40% (P=0.013)

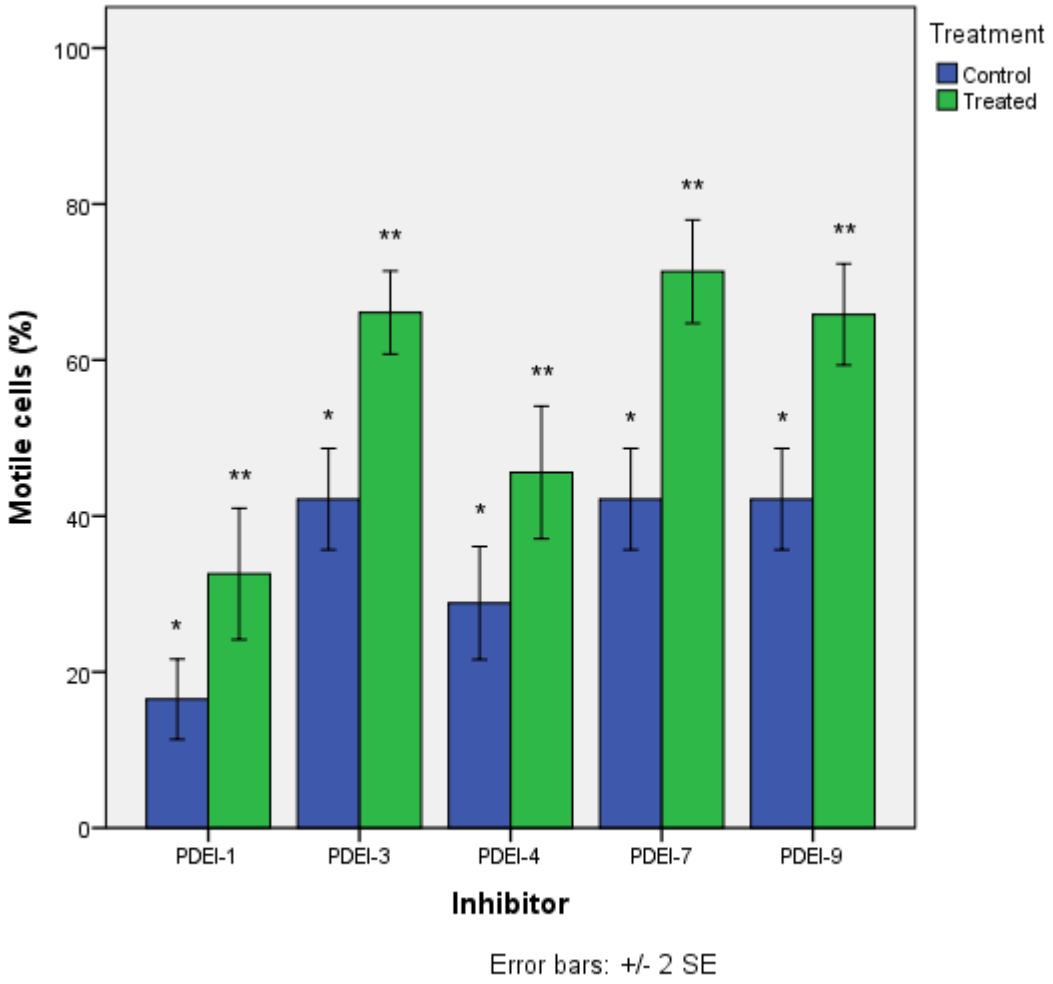

Figure 26. Figure demonstrating the effect of PDEI-1 (8-MeOM-IBMX). PDEI-3 (Milrinone), PDEI-4 (Rolipram) PDEI-7 (BRL) and PDEI-9 (Papaverine) on the % motile cells in the 40 fraction of patient samples (n=3). There was a significant difference between control and treated using all inhibitors:

PDEI-1 (P<0.000)

PDEI-3 (P<0.000)

PDEI-4 (P=0.008)

PDEI-7 (P<0.000)

PDEI-9 (P<0.000)

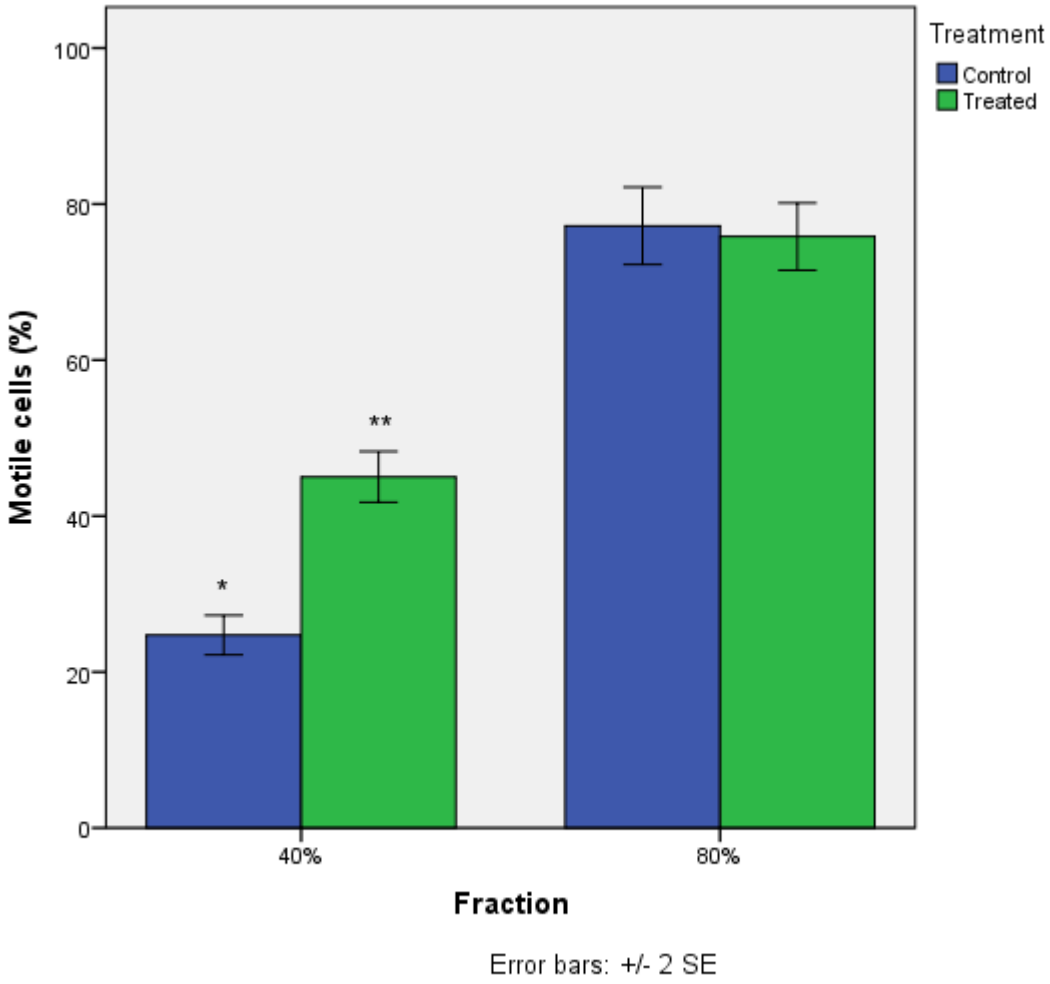

Figure 27. Figure demonstrating the effect of PDEI-4 (Rolipram) on the % motile cells in the 40 and 80% fractions of patient IVF samples (n=33). There was a significant difference between the 40% fraction but not the 80%:

40% (P<0.000)

80% (P=0.543)

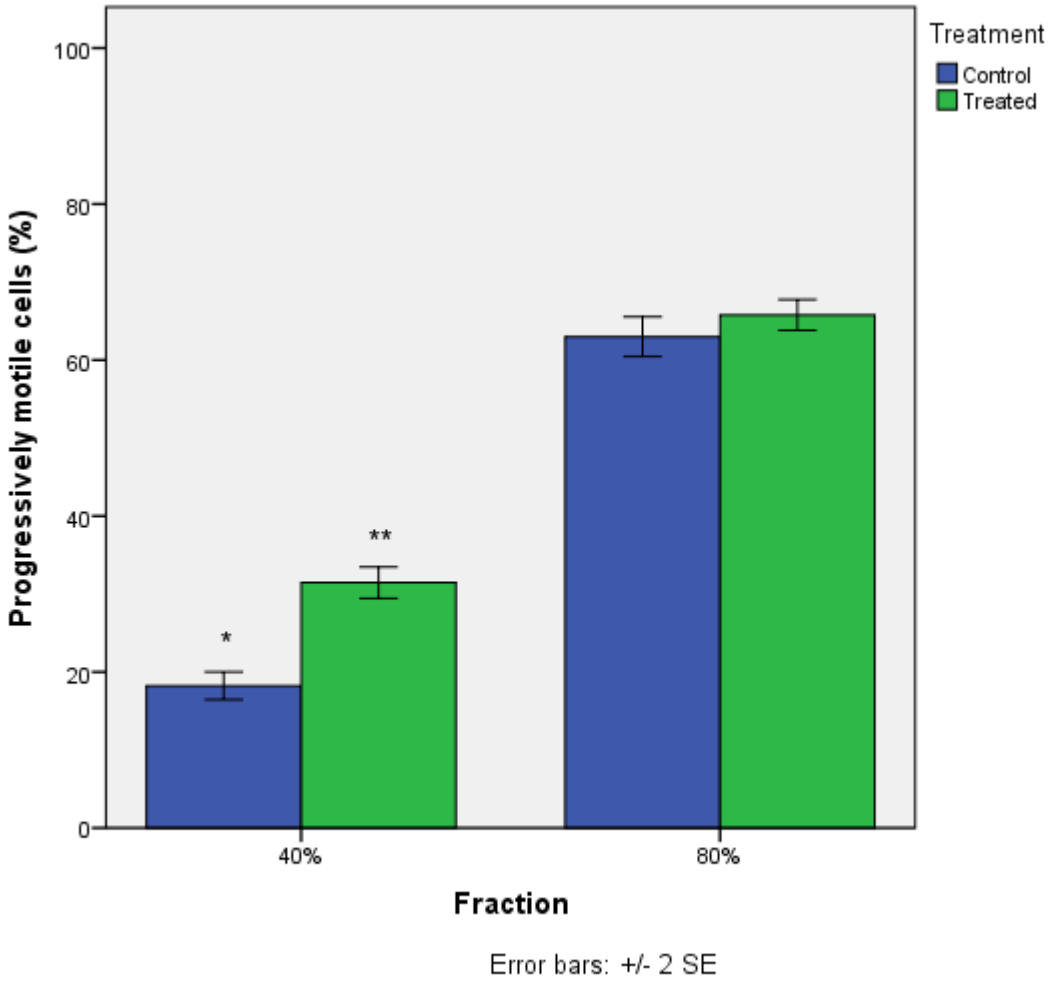

Figure 28. Figure demonstrating the effect of PDEI-4 (Rolipram) on the % progressively motile cells in the 40 and 80% fractions of patient IVF samples (n=33). There was a significant difference between the 40% fraction but not the 80%:

40% (P=0.012)

80% (P=0.141)

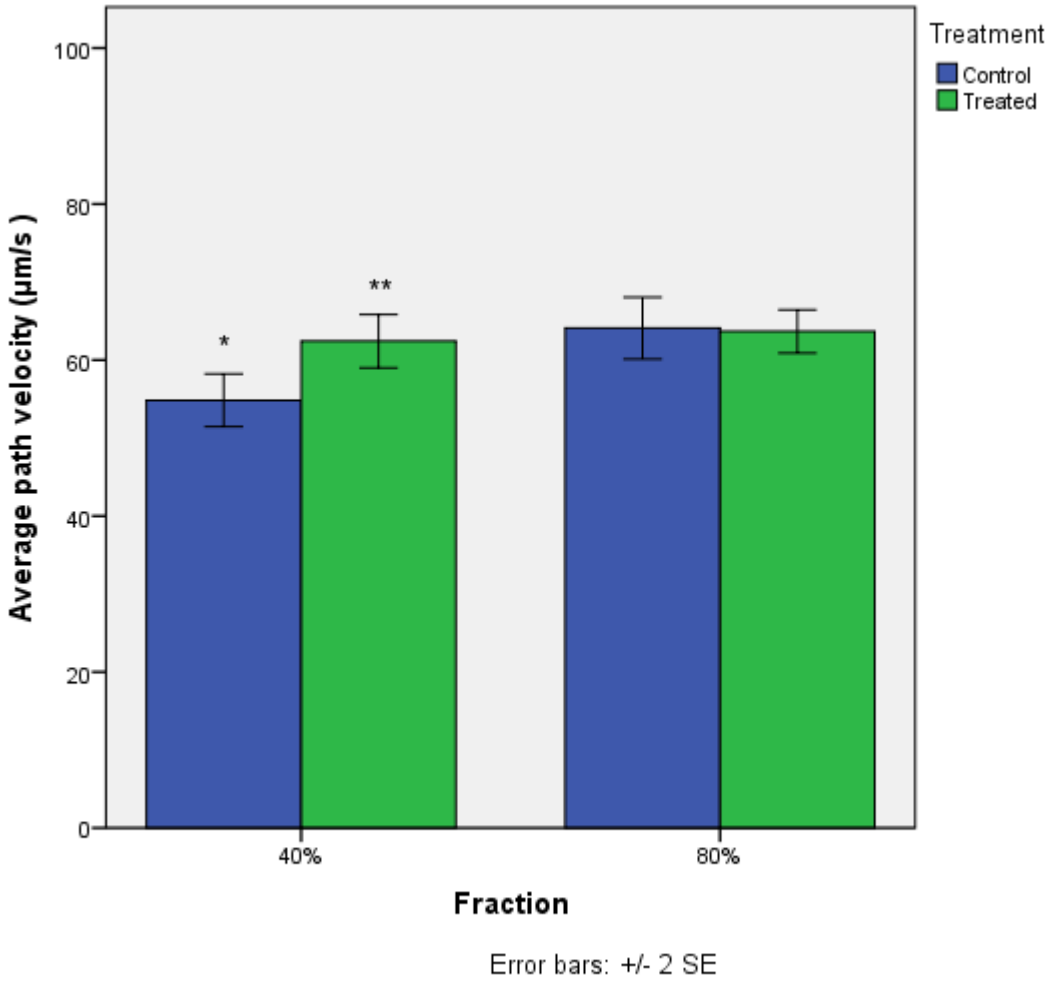

Figure 29. Figure demonstrating the effect of PDEI-4 (Rolipram) on the average path velocity in the 40 and 80% fractions of patient IVF samples (n=33). There was a significant difference between the 40% fraction but not the 80%:

40% (P=0.018)

80% (P=0.826)

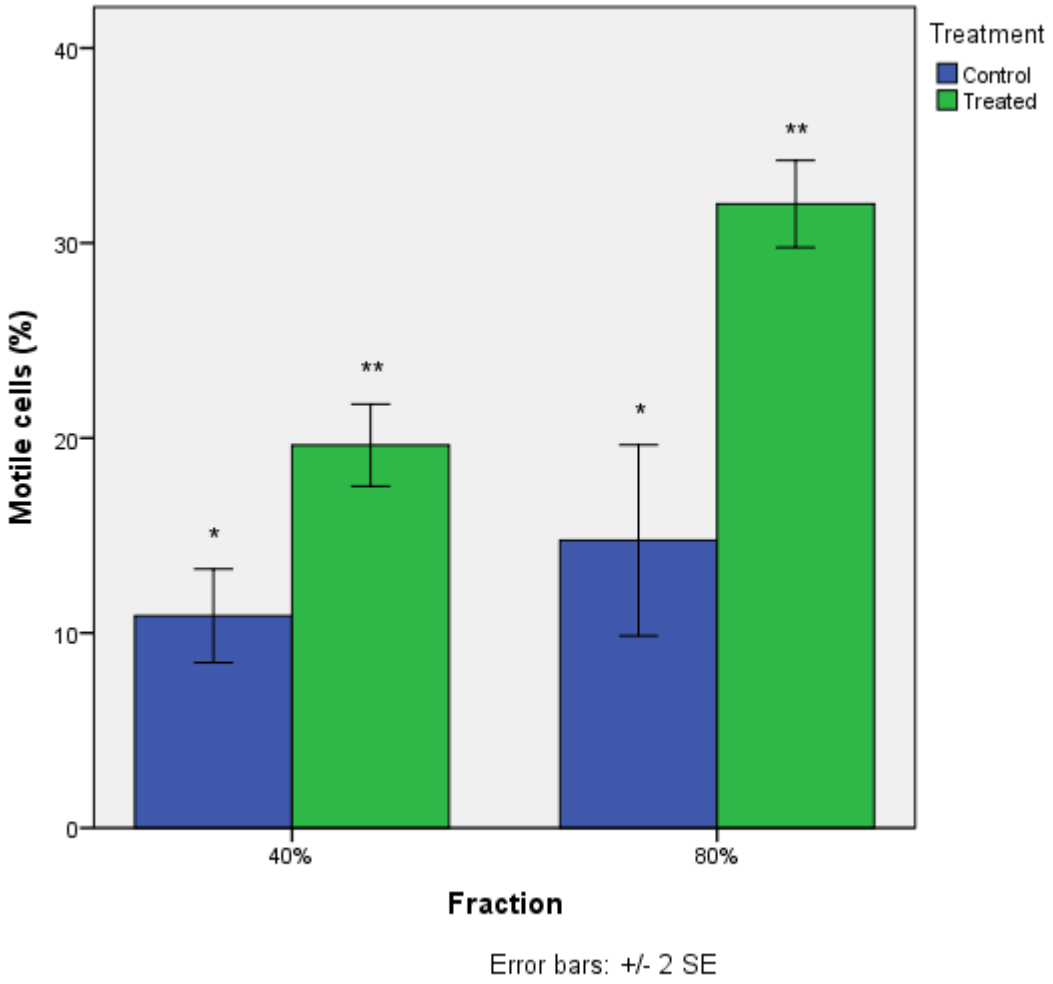

Figure 30. Figure demonstrating the effect of PDEI-4 (Rolipram) on the % motile cells in the 40 and 80% fractions of patient ICSI samples (n=27). There was a significant difference between the 40% fraction and the 80%:

40% (P<0.001)

80% (P<0.001)

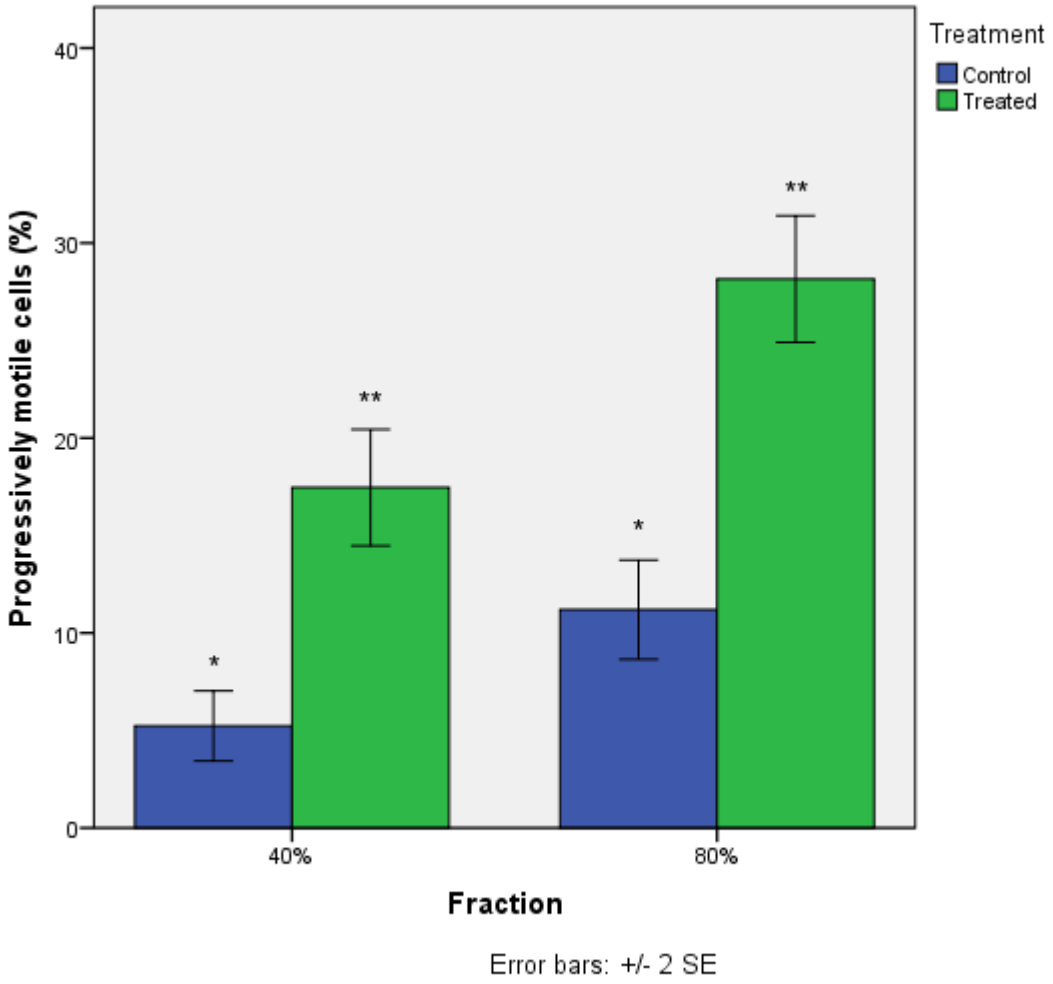

Figure 31. Figure demonstrating the effect of PDEI-4 (Rolipram) on the % progressively motile cells in the 40 and 80% fractions of patient ICSI samples (n=27). There was a significant difference between the 40% fraction and the 80%:

40% (P<0.001)

80% (P<0.001)

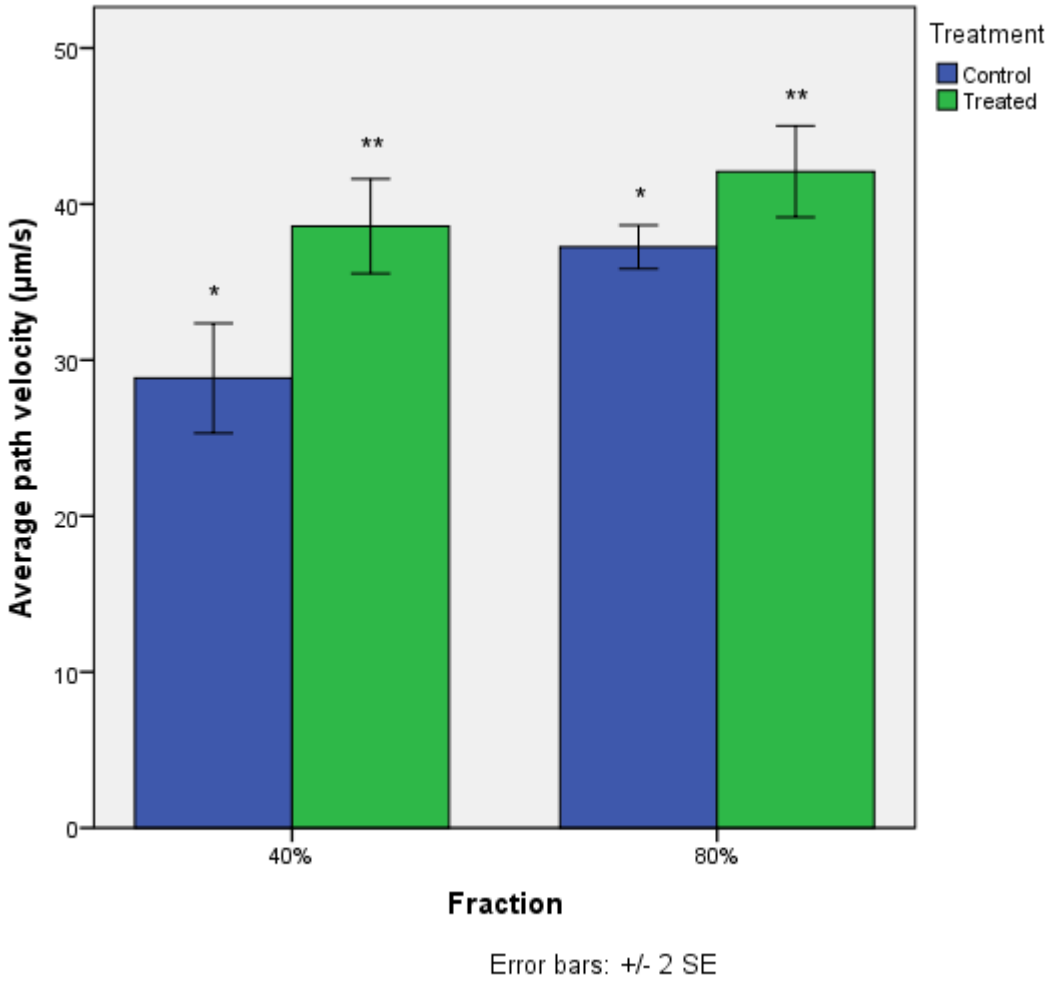

Figure 32. Figure demonstrating the effect of PDEI-4 (Rolipram) on average path velocity in the 40 and 80% fractions of patient ICSI samples (n=27). There was a significant difference between the 40% fraction and the 80%:

40% (P<0.001)

80% (P<0.001)

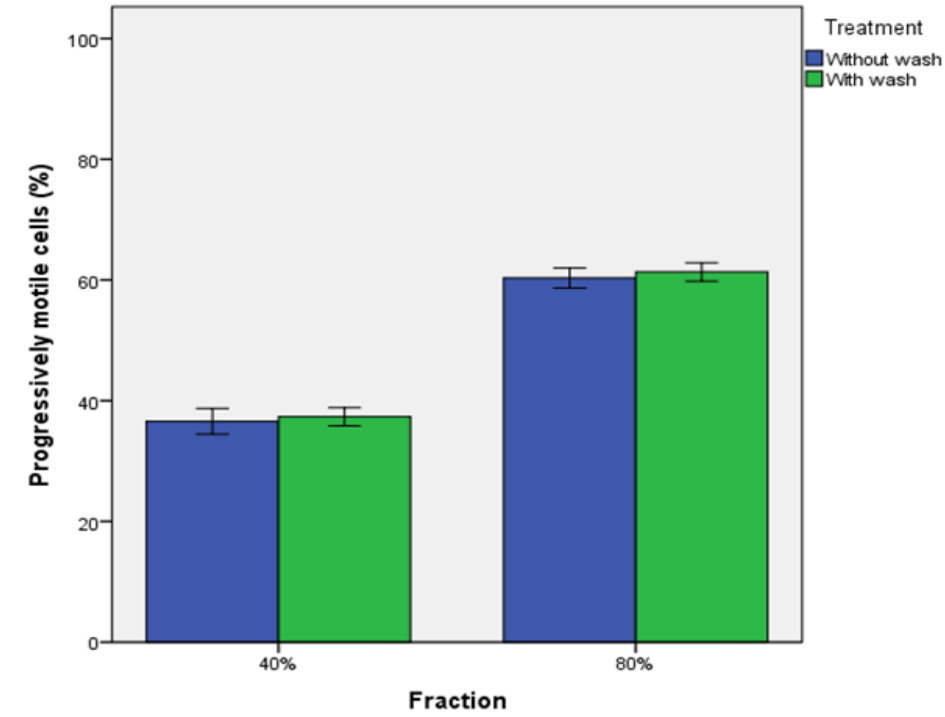

Figure 33. Figure demonstrating the effect of an additional wash step with PDEI-4 (Rolipram) on the % motile cells in the 40 and 80% fractions of patient samples (n=3). There was no significant difference

40% (P=0.179)

80% (P=0.896)

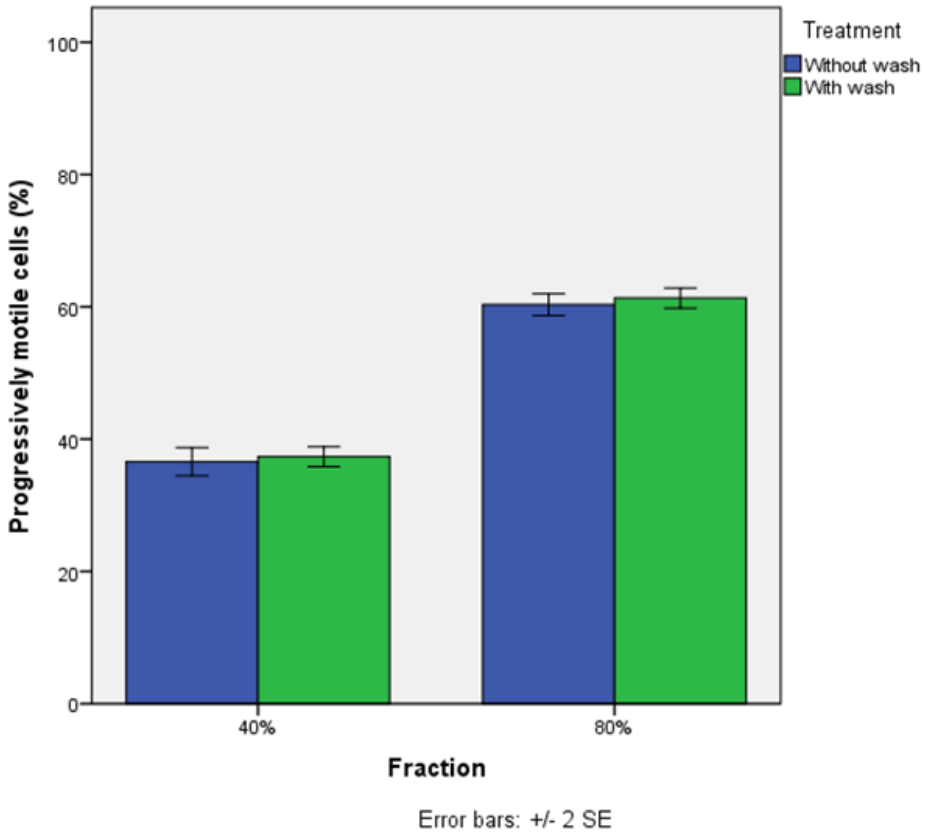

Figure 34. Figure demonstrating the effect of an additional wash step with PDEI-4 (Rolipram) on the % progressively motile cells in the 40 and 80% fractions of patient samples (n=3). There was no significant difference

40% (P=0.476)

80% (P=0.483)

CASA

The following data demonstrates the reliability and reproducibility of using CASA throughout experiments. It is recognised that a number of variables exist with the use of CASA which may affect the overall result. The following results serve as a control for this study.

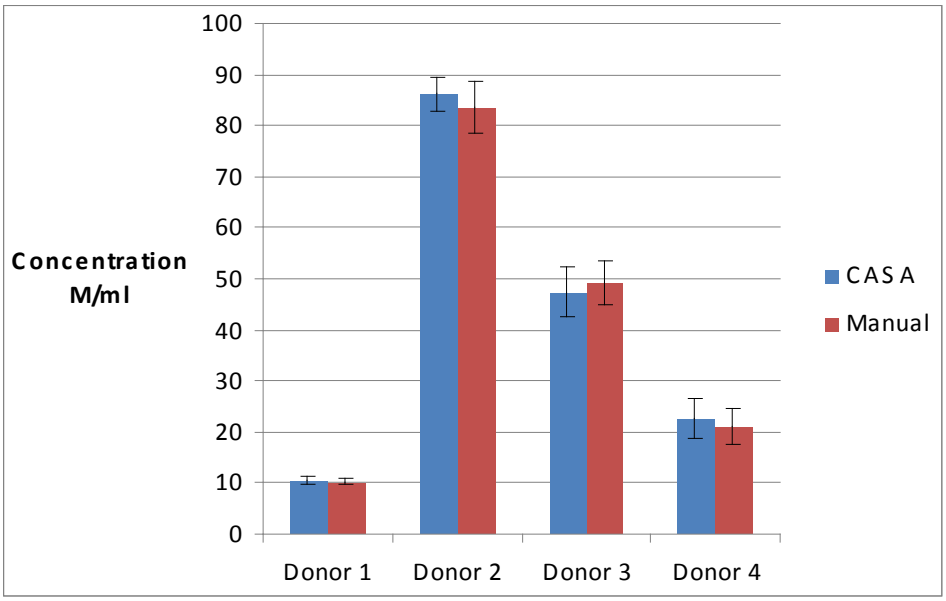

Figure 35. Graph demonstrates a comparison of concentration counts using CASA or haemocytometer. Error bars of standard deviation represent four counts of a minimum of 800 cells. There were no significant differences between counts for each donor (student's paired t-test P=0.983, 0.735, 0.834 and 0.896).

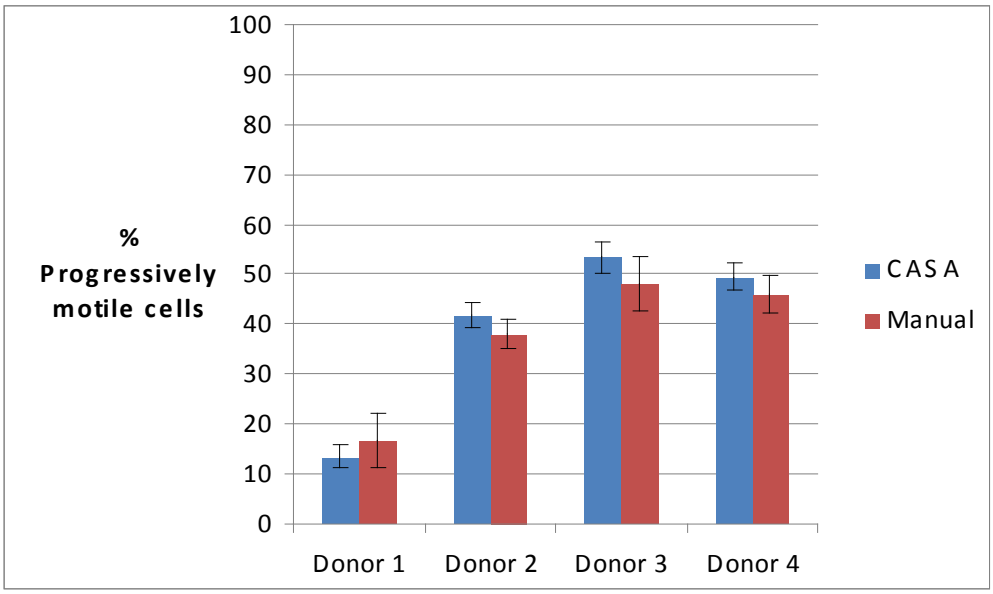

Figure 36. Graph demonstrates a comparison of progressive motility counts using CASA or haemocytometer. Error bars of standard deviation represent four counts of a minimum of 800 cells. There were no significant differences between counts for each donor (student's paired t-test P=0.785, 0.587, 0.564 and 0.649).